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(54) GENE PROTEIN PRODUCT AND TREATMENT OF CELL

(57)Abstract:

PURPOSE: To obtain a substantially purified, loss-free and biochemically active protein having high potency by introducing a genetic substance into an insect cell cultured product and culturing the produced genetic protein.

CONSTITUTION: A genetic substance is introduced through a virus vector or the like into an insect cell cultured product to produce a gene protein. The gene protein product obtained by culturing the gene protein functions at cellular level or subcellular level when introduced into a target cell.

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CLAIMS

[Claim(s)]
[Claim 1] In the approach of producing the gene production polypeptide related to a predetermined gene refined substantially Choose the vector which conveys cDNA which has the nucleotide sequence which is substantially equivalent to a nucleotide sequence in this gene, insert this DNA in this vector, and this vector is introduced into a cell culture object. The polypeptide production approach which consists of forming the manifestation system which spreads this polypeptide, growing up this cell, disuniting this cell, and extracting this polypeptide from this cell, and refining this polypeptide.
[Claim 2] The approach according to claim 1 this vector is a virus.
[Claim 3] The approach according to claim 1 this virus is a baculovirus.
[Claim 4] This virus is Autographa. The approach according to claim 1 of being a californica nuclear polyhedrosis virus.
[Claim 5] The approach according to claim 1 this cDNA has a PORIHE drine compounds gene promotor property.
[Claim 6] The approach according to claim 1 of this cDNA having the promotor of a PORIHE drine compounds gene, and inserting this cloning cDNA in this promotor's lower stream of a river.
[Claim 7] The approach according to claim 1 this cell is an eukaryote cell.
[Claim 8] This cell is Spodoptera. The approach according to claim 1 of being a frugiperda cell.
[Claim 9] The method according to claim 1 of carrying out this purification by the immunity affinity chromatography method.
[Claim 10] The method according to claim 1 of guiding this cDNA from an eukaryote gene.
[Claim 11] The method according to claim 1 of guiding this cDNA from an eukaryote antioncogene.
[Claim 12] The method according to claim 1 of guiding this cDNA from a retinoblastoma gene.
[Claim 13] The polypeptide manifestation system which consists of a vector for conveyance, and a cell culture object in the polypeptide manifestation system which discovers the gene production polypeptide related to a predetermined gene.
[Claim 14] The manifestation system according to claim 13 this whose vector is a virus.
[Claim 15] The manifestation system according to claim 13 this whose vector is a baculovirus.
[Claim 16] This vector is Autographa. System according to claim 1 which is a californica nuclear polyhedrosis virus.
[Claim 17] The system according to claim 13 whose cell of this cell culture object is an insect cell.
[Claim 18] This cell is Spodoptera. System according to claim 13 which is a frugiperda cell.
[Claim 19] With regards to a predetermined gene, choose the vector which conveys cDNA which has the nucleotide sequence which is substantially equivalent to a nucleotide sequence in this gene, insert this DNA in this vector, and this vector is introduced into a cell culture object. The polypeptide which the manifestation system which spreads this polypeptide is formed, this cell was grown up, and this cell was disunited, and extracted this polypeptide from this cell, and was obtained by the approach of consisting of refining this polypeptide and which was refined substantially.
[Claim 20] A polypeptide including guiding this cDNA from a retinoblastoma gene according to claim 19.
[Claim 21] The therapy approach which consists of checking this deficit, a mutation gene, or that there is no gene in the approach of having a deficit antioncogene and a mutation antioncogene or treating a cell without a gene, and asking for an opposite part without the deficit which is the natural student gene or its clone of this gene, and producing the protein production object of the gene of an opposite part.
[Claim 22] An approach including this conveyance carrying out the microinjection of this protein production object

:o this cell according to claim 21.
[Claim 23] The approach according to claim 21 this purification includes the dialysis to the buffer solution of this protein.
[Claim 24] This buffer solution is tris. - Approach containing HCl, KCl, EDTA, DTT, and glycerol according to claim 21.
[Claim 25] This tris - Approach according to claim 24 pH of HCl is 7.4.
[Claim 26] This buffer solution is KCl of DTT of the one section, EDTA of the one section, and the 100 sections, and tris of the 200 sections. - Approach containing HCl according to claim 23.
[Claim 27] An approach given in claim 21 term in which this check investigates the existence of a protein production object, and measures the amount of protein production.
[Claim 28] An approach including determining whether this measurement uses a specific antibody for this protein, and this antibody and the immune complex of this protein form according to claim 21.
[Claim 29] The drugs constituent which serves as a polypeptide as an active ingredient from suitable support physiologically.
[Claim 30] The drugs constituent according to claim 29 this whose polypeptide is pp110RB.
[Claim 31] The drugs constituent according to claim 29 which chooses this active ingredient from the protein production object of a milk antioncogene, a Wilms' tumor inhibitor, a Beckwith-Wiedemann syndrome inhibitor, a transition sexual cell neoplasm inhibitor, a neuroblastoma inhibitor, a small cell lung cancer inhibitor, a kidney androblastoma inhibitor, and a colorectal cancer inhibitor.
[Claim 32] A drugs constituent with the following amino acid sequences of this active ingredient according to claim 29.
[Formula 1]

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M P P K T P R K T A A T A A A A A A E P P A P P P P P P P E E D P E ( 34)
Q D S G P E D L P L V R L E F E E T E E P D F T A L C Q K L K I P D M V R E R A ( 74)
W L T W E K V S S V D G V L G G Y I Q K K K E L W G I C I F I A A V D L D E M S (114)
F T F T E L Q K N I E I S V H K F F N L L K E I D T S T K V D N A M S R L L K K (154)
Y D V L F A L F S K L E R T C E L I Y L T Q P S S S I S T E I N S A L V L K V S (194)
W I T F L L A K G E V L Q M E D D L V I S F Q L M L C V L D Y F I K L S P P M L (234)
L K E P Y K T A V I P I N G S P R T P R R G Q M R S A R I A K Q L E N D T R I I (274)
E V L C K E H E C N I D E V K N V Y F K N F I P F M N S L G L V T S N G L P E V (314)
E N L S K R Y E E I Y L K N K D L D A R L F L D H D K T L Q T D S I D S F E T Q (354)
R T P R K S N L D E E V N V I P P H T P V R T V M N T I Q Q L M M I L N S A S D (394)
Q P S E N L I S Y F N N C T V N P K E S I L K R V K D I G Y I F K E K F A K A V (434)
G Q G C V E I G S Q R Y K L G V R L Y Y R V M E S M L K S E E E R L S I Q N F S (474)
K L L N D N I F N M S L L A C A L E V V M A T Y S R S T S Q N L D S G T D L S F (514)
P W I L N V L N L K A F D F Y K V I E S F I K A E G N L T R E M I K H L E R C E (554)
H R I M E S L A W L S D S P L F D L I K Q S K D R E G P T D H L E S A C P L N L (594)
P L Q N N H T A A D M Y L S P V R S P K K K G S T T R V N S T A N A E T Q A T S (634)
A F Q T Q K P L K S T S L S L F Y K K V Y R L A Y L R L N T L C E R L L S E N P (674)
E L E M I I W T L F Q H T L Q N E Y E L M R D R H L D Q I M M C S M Y G I C K V (714)
K N I D L K F K I I V T A Y K D L P H A V Q E T F K R V L I K E E E Y D S I I V (754)
F Y N S V F M Q R L K T N I L Q Y A S T R P P T L S P I P H I P R S P Y K F P S (794)
S P L R I P G G N I Y I S P L K S P Y K I S E G L P T P T K N T P R S R I L V S (834)
I G E S F G T S E K F Q K I N Q M V C N S D R V L K R S A E G S N P P K P L K K (874)
L R F D I E G S D E A D G S K N L P G E S K F Q Q K L A E M T S T R T R M Q K Q (914)
K M N D S M D T S N K E E K (928)
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[Translation done.]

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] On the whole, this invention relates to the cell therapy for treating the production approach of a gene protein production object, and a cell, and controlling tumorigenesis. This invention relates to the cell therapy method which controls a cell therapy and tumorigenesis again. This invention is government support license number EY05758) invention, and is invention attained in collaboration with national American Insurance Association (the National Institute of Health) and a cull FORUNIA university. Therefore, about this invention, the government also has a fixed right.
[0002]
[Description of the Prior Art] There is research quite detailed about the tissue of Homo sapiens or an animal, and much deficits are checked on cell level. Many of these deficits have a hereditary cause, and most depends it on a deficit gene. A deficit may consider point mutation etc. as a cause, and as a result, the nucleotide sequence of the gene itself may divide, or it may show an unusual change. When there is a malfunction gene, the gene protein production object with which it becomes impossible to have produced the gene protein production object finally, and itself suffered a loss will be produced.
[0003] A gene therapy can be applied when identifying a deficit gene about both Homo sapiens and an animal. In this case, what is necessary is to lead to the nucleus of the cell which should treat a cloning gene, and just to correct an unusual genetic material or a deficit genetic material. The matter used for this approach has a high manufacturing cost, and needs a complicated elaborate experimental device, and needs application of a complicated elaborate genetic manipulation technique. Generally such a technique cannot be used, therefore only the laboratory where most treats molecule genetic manipulation comparatively elaborate to a small number of altitude can be used.
[0004] Costs of a gene therapy are high, and since it generally cannot use, another cell therapy is searched for. It is possible to draw a conclusion from analysis of the molecular structure and the function of a protein production object about the insurance of the gene which produces this production object. For example, what is necessary is to use a gene protein production object in many cases, and just to opt for the interaction between genes, in order to determine the neoplasm control mechanism of the body. What is necessary is just to refer to the parent-patent application specification of this application about a neoplasm control mechanism. the top where it is reliable and cost is low in order to solve a gene function and a gene interaction -- dependability -- the approach of producing the gene protein production object which can offer a lot of [possible / prediction] high protein is very desirable.
[0005] Not only a gene protein production object is useful, but [although a gene function and a gene interaction are solved] this protein itself can use it for the therapy of a deficit gene condition. In this case, it is advantageous to introduce the suitable gene protein production object for a cell with a deficit genetic material, and it is effective. Cost is low and sometimes easier than the case where the direction of installation of a protein production object prescribes the genetic material itself for the patient for the purpose of a therapy by the case.
[0006] What is necessary is just to refer to the patent application specification submitted to this application and coincidence about a protein therapy.
[0007] When it sees from the recognition about the importance of a gene protein production object, it is very advantageous that the approach of preparing and separating a gene protein production object in the form refined substantially can be used now. in order that that the activity matter can be used as it is so much biochemically may study the biochemical property and molecule behavior which are participating in the heredity mechanism -- a therapy sake -- an owner -- it is a meaningless advance.

[0008] Generally, in production of a different laboratory scale from the case of extensive production, the gene protein production object is produced also by the synthetic production only from the cell. Speaking of the induction from a cell, little existence of the cell protein is recognized very much. Consequently, the attempt which guides sufficiently a lot of protein from the source of nature is not realistic.
[0009] Speaking of the synthetic producing method, the attempt which introduces the decode array of a gene into a bacteria expression vector, and discovers protein was not successful except for some cases. Bacteria production protein has weak solubility. The point of giving the result which a bacterial cell could not change eukaryote protein, and analysis of such protein mistook when modification after a translation was required about protein has another fault which uses a bacteria manifestation system. That is, generally bacteria production protein has low solubility, and since there is a molecule deficit, usefulness has a limit.
[0010] It not only cannot produce enough a lot of protein, but by the usual laboratory-approach which makes a protein production object, the purity of production protein is not enough, either and variation is in purity. TrpE-RB fusion protein may be discovered for the example of representation of the difficulty at the time of producing a certain kind of protein production object, T7 RNA polymerase discovered to E.coli may be used for it, and a polypeptide may be produced. These approaches are comparatively complicated and need complicated elaborate biochemical actuation. Furthermore, since the target polypeptide does not carry out little deer production very much, these approaches have a big limit. Moreover, the polypeptide produced by this approach is seen in molecule, and it is common not to be desirable. For example, it is because this is not phosphoryl-ized.
[0011] Therefore, biochemical [desired] and the desired approach of producing a polypeptide with a biophysical property in a substantial amount are strongly desired from the importance of a gene production object polypeptide.

[0012] A remarkable advance is seen about an understanding of the function of the gene in living body health maintenance. If that it can generally say cannot produce protein with proper cytogene, various kinds of abnormalities will occur to a living body. It can ask that the whole gene is lost or the gene itself is missing by various kinds of causes for this reason that cannot be produced. From recognition of these factors, remarkable progress is accepted in a gene therapy.
[0013] For example, it is thought that the Wilms' tumor which is infantile kidney cancer is produced by gene inactivation of a chromosome 11. It is proved that oncogenicity can be controlled, if the normal chromosome 11 is introduced into a Wilms' tumor cell when the minute cell fusion medium transferring method of a single strand chromosome is used. On the other hand, this effectiveness is not accepted in Chromosome X and installation of 13.
[0014] On experiment level, although a certain extent of transition of a human chromosome without a deficit is useful, such transition is not applicable to the therapy of a genetic defect. Preparation of the suitable chromosome for a therapy not only needs remarkable skill, but requires time amount for one upwards, and it is because cost is high. For this reason, in many cases, this approach is nonpermissible.
[0015] When it is not desirable to use a chromosome without a deficit for a therapy, thinking theoretically below is introducing into a patient the proper whole gene or the part which has the effect at least. Although this approach has workability higher than the approach of introducing a chromosome without a deficit, it is only a certain fixed case that a gene therapy is desired.
[0016] Not only cost is very high, but separation of the proper nucleic-acid matter, sequencing, and cloning require time amount about this point. In addition, the complicated elaborate molecule genetic manipulation approach which can be used only in the location restricted very much also in the world is required of such an approach. Furthermore, such an approach produces a lot of matter suitable for a therapy even now.
[0017] As mentioned above, a specific therapy of the cell level which cost is comparatively low, and it is reliable, and can more generally use by using the bionics-approach, and uses the specific matter in a biochemical operation is desired strongly. Furthermore, a production object effective in a therapy is introduced on cell level, and a therapy which brings about change of neoplasm control etc. is also desired strongly. Although it is needless to say, it is strongly wished also about the production object which can produce so much in the state of purification without variation, and can be introduced into a deficit cell easily and effectively.
[0018] It is in the 1st purpose of this invention offering a specific therapy safe on the whole and and a production object effective in controlling cancer control.
[0019] It is in another purpose of this invention offering a cancer inhibitory-control production object and a therapy effective in the control and extermination using the bionics-approach and a production object of a cancer

tumor. Moreover, it is also the purpose of this invention to offer the drugs constituent for cancer treatment which demonstrates an operation on cell level and the level between cells.
[0020] It is in still more nearly another purpose of this invention offering the drugs constituent which treats the symptom produced by the absence of the deficit or mutation antioncogene which makes nature or a synthetic production object an active ingredient, and an antioncogene.
[0021] This invention leads a specific antioncogene protein production object to an object cell, and consists of a cell therapy which realizes cancer control.
[0022] This invention offers the conventional radiotherapy and the cure for cancer with little need for a chemotherapy. Furthermore, this invention approach can be applied after discovery of disposition which is easy to suffer from cancer hereditarily at the very early stage before tumorigenesis begins.
[0023] this invention -- an antioncogene protein production object -- good facilities -- and in the point of using it at comparatively low cost and realizing cancer control on cell level, it is very advantageous.
[0024] Although there is a point which has not been clearly carried out about whether cancer will be generated enough when the antioncogene beyond one or it in a certain cell inactivates then, cell installation of a gene protein production object is new approach, and is the advantageous approach to a malignant tumor therapy. This invention is in the condition which suppressed the damage to a living body to the minimum unlike the cytotoxic cancer therapy from the former, and is still more advantageous in the point of bringing about an advantageous change on cell level.
[0025] As mentioned above, it is in the 1st purpose of this invention offering the approach of producing an activity gene production polypeptide without the deficit of a substantial amount.
[0026] Furthermore, another purpose of this invention has structure and a function in offering the same production approach of a specific gene production polypeptide as a natural student polypeptide.
[0027] If it summarizes, the above-mentioned purpose and other purposes of this invention are realizable by the approach of producing a substantial quantity of a request polypeptide by introducing a genetic material into an insect cell. For example, if a cloning gene, gene fragmentation, or its derivative is introduced into a host cell using a suitable vector, it is a substantial amount and the protein of a high grade can be produced with a high level.
[0028] This invention produces the polypeptide of high quality in a substantial amount, offers the approach of carrying out things of investigating a gene function on cell level, and is very advantageous.
[0029] Moreover, this invention is advantageous, is reliable, moreover, can produce this polypeptide at repetitive comparatively low cost, and is very advantageous also in this point.
[0030] Furthermore, this invention can produce the high quality polypeptide of a considerable amount which can solve an intergenic interaction on cell level and the level between cells, and is advantageous also at this point.
[0031]
[Easy explanation of a drawing]
Other purpose, descriptions, and these implementation approaches should become clear from the following publications at the above-mentioned above-mentioned purpose of this invention and the description, and the list. Moreover, it refers to an understanding of this invention itself at an accompanying drawing, and it should be obtained if the publication about the example of invention is read. Drawing 1 is the schematic diagram showing the configuration of the Baculoviridae manifestation vector which compounds pp110RB. Drawing 2 is the western blotting (Western blot) of a ppRB infection insect cell. Drawing 3 is western blotting which shows the cell extract extracted from the infected cell even 72 hours after infection. Drawing 4 is a microphotography in which the normal position between cells of RB protein is shown. Drawing 5 is the microphotography of infection Sf9 cell. Drawing 6 is a radioautograph which shows the result of phosphoryl-izing of RB protein in an insect cell, and dephosphorylation analysis. drawing 7 -- pMG 3-245 -- anti- -- it is drawing showing the rough lysate from -RB, nfection Sf9 cell, and the electrophoresis analysis result of eluate. Drawing 8 shows the fusion protein and the south Western (Southwestern) DNA binding measuring method of Baculoviridae-manifestation pp110RB which were applied to SDS-PAGE and a Coomassie-brilliant-blue-staining method 10%. It is the radioautograph of the blot from parallel gel to the gel used in order that drawing 9 might create drawing 8 , and the incubation of this blot is carried out by the 32P-labeling DNA fragment. It is the chromatogram which shows complexing of drawing 10 Baculoviridae-manifestation RB protein and an SV40 T antigen. The nuclear transformation of the purification RB protein after the microinjection to the cytoplasm of drawing 11 Saos-2 cell is shown.
[0032]
[The best example of invention] All have adopted the contents here and the reference which makes reference on

:hese specifications constitutes a part of this specification publication. Hereafter, this invention will be explained in :he following order.
A. an outline -- the nuclear transformation B7. epitome [0033] of specific complex formation B6. purification RB protein with the RB protein purification B5.DNA-avidity from the phosphoryl-ized B4. infection insect cell after the nuclear normal position of the manifestation B3. extraversio RB protein of the extraversio RB protein in the configuration B-2. infection insect cell of example of B.RB gene production object B1. recombination Baculoviridae, and a translation, and an SV40 T antigen A. When outline this invention introduces a genetic material into an insect cell culture object, it can be used for this gene protein producing a specific gene protein production object by culture about the approach of producing gene protein. If this approach is used, the deficit with a high force value refined substantially can twist, and activity protein can be obtained biochemically.
[0034] The manifestation system of this invention has a large application. That is, for example, Homo sapiens, the cloning gene of an animal, its fragmentation, a homologue, a derivative, or a part is applicable to production of a request protein production object. Thus, needless to say, the produced protein is applicable to the therapy of a deficit cell at the elucidation of a gene function. a flume -- obtaining -- it is because a gene interacts on cell level.

[0035] Since the insect cell culture object had an eukaryote property when a gene protein production object was produced, the suitable thing was found out. In order to introduce a genetic material into a cell culture object, the usual vectors, such as a virus vector, can be used.
[0036] For example, in order to produce RB gene protein production object, the virus vector of an insect cell culture object is used. In order to rearrange with a high level in a culture insect cell and to produce protein about this point, Autographa KARIHORUNIKA of Baculoviridae (Autographa californica), i.e., a nuclear polyhedrosis virus, (AcNPV) is used as a virus expression vector independent of a helper. This virus breeds in the cultivated matrix caterpillar cabbage armyworm from the United States, i.e., a Spodoptera full GIPERUDA (Spodoptera frugiperda) (Sf9) cell. This virus has the promotor with a powerful polyhedron gene adjusted temporarily, and that production object occupies 50% or more of all cell protein at the time of lytic infection. By in vivo recombination, the decode array of a foreign gene receives the translational control of a polyhedrin promotor, and, as a result, discovers protein with a high level. Furthermore, it doubles correctly and the protein which carried out in this way and was produced includes the same modification after a translation as the protein looked at by the original high-class eukaryote.
[0037] This invention is a cell therapy which introduces a specific antioncogene protein production object into an object cell, and realizes neoplasm control. Since a given cell has a deficit gene, in the point which causes the protein manifestation deficit in a cell, it may be missing.
[0038] It is related with this invention approach using the gene protein production object related to a deficit gene. A purification protein production object is introduced into an object cell, for example, neoplasm control is realized. By supporting and introducing this production object into support suitable in pharmacology, this protein production object is cell level, or acts on the bottom level of a cell.

[0039] the cell RB gene protein which whose RB gene which is an antioncogene is missing as an example of this approach, or does not have it was boiled and introduced. What is necessary is just to refer to the parent-patent application specification which has made reference on these specifications about the detailed information about RB gene.
[0040] In the suitable example, the small-child cancer which is a retinoblastoma, i.e., a symptom with the rare retina under growth, as a PM of recessive tumorigenesis research is used. Based on the normal position and its recessive nature of the heredity element to a chromosome 13q14 which is involving, the gene presumed to be an antioncogene, i.e., a retinoblastoma susceptibility gene, (RB) was cloned. A 4.7 kilobase mRNA imprint is discovered at all in the normal organization which the exon of 27 distributed this gene to the genomic DNA of 200 kilobases, and inspected. By the sequence analysis of a complementary DNA clone, the long open reading frame which has coded the hypothetical protein of the amino acid of 928 became clear. When the antibody to the predetermined epitope guessed from the RBcDAN array was used, as for this RB gene production object, relative molecular weight (Mr) was identified the nucleus phosphoprotein of 110,000-114,000. This was named pp110RB.
[0041] only to a retinoblastoma, it has become clear that the deficit of RB gene function is boiled also at the neoplasm of some of others including a breast cancer, an osteosarcoma, a prostatic cancer, or small cell lung cancer again, and is involving. According to the latest research, if RB gene is reintroduced into a retinoblastoma, an osteosarcoma, or a prostate gland cancer cell by retrovirus medium gene transition, it will have become clear

that some fields of new phenotypes including the tumorigenesis in a nude mouse are controlled clearly. This is a direct proof of the neoplasm control function of RB gene. However, the foundation in the molecular level of this biological activity is not established yet.

[0042] Although all the biochemical properties and biological functions of an antioncogene production object are not solved the place to current, it is because there is little this protein in a cell as for this, so it is difficult to obtain in sufficient amount. What is necessary is just to refer to the parent application specification (the name of invention: the production approach of a gene protein production object) for which it applied to this application and coincidence about this point.

[0043] The cell therapy which has a deficit gene or introduces a specific gene protein production object into a cell without a gene was invented. According to this invention, the deficit refined substantially twists and the proper amount of activity gene production protein can be biochemically introduced into a deficit gene with an effective dose on a therapy.

[0044] The cell therapy of this invention has a large application application. This protein production object is useful not only to the therapy of a deficit cell but the elucidation of a gene function. It is because a gene interacts on cell level.

[0045] In the one specific example, a retinoblastoma gene protein production object, i.e., pp110RB, has a deficit gene, or it can apply it to the therapy of an eukaryote cell without a gene widely.

[0046] In addition, according to this invention, completely like Shinsei Homo sapiens pp110RB, purification protein combined DNA and has formed the SV40 T antigen and the specific complex. Moreover, according to the proteinic nuclear transformation immediately performed after the microinjection, it became clear that this protein showed activity and could use it for a therapy.

[0047] B. Explain in more detail about the example of RB gene production, next the production approach by this invention of pp110RB protein. What is necessary is just to refer to the following parent-patent application specifications about information detailed to the pan about this protein and a retinoblastoma gene.

[0048] Although all the biochemical property and biological functions of an antioncogene production object, such as RB gene, are not solved, this is because it is difficult to obtain purification protein in sufficient amount. That is, it is because it is because a cell has little protein and the protein manifestation by the installation to the bacteria expression vector of the coding array of a gene has not succeeded in one except for a part in addition again. It has been concluded that the problem which the approach used now has from these was avoidable if a cloning gene is discovered in an eukaryote system. Although it will be an antioncogene if this invention is illustrated, although the specific example of this invention is related with production of RB gene protein production object, it can apply also to production of the protein production object of other eukaryote genes which are not restricted to this, therefore is related also with production of such a protein production object.

[0049] In order to produce recombination protein with a high level in a culture insect cell, it is desirable although Autographa KARIHORUNIKA of Baculoviridae (Autographa californica), i.e., a nuclear polyhedrosis virus, (AcNPV) is used as a virus expression vector independent of a helper. This virus breeds in the cultivated matrix caterpillar cabbage armyworm from the United States, i.e., a Spodoptera full GIPERUDA (Spodoptera frugiperda) (Sf9) cell. This virus has the promotor with a powerful polyhedron gene adjusted temporarily, and that production object occupies 50% or more of all cell protein at the time of lytic infection. By in vivo recombination, the decode array of a foreign gene receives the translational control of a polyhedrin promotor, and, as a result, discovers protein with a high level. Furthermore, it doubles correctly and the protein which carried out in this way and was produced includes the same modification after a translation as the protein looked at by the original high-class eukaryote.

[0050] In order to test the manifestation possibility of functional RB protein by the Baculoviridae system, cloning Homo sapiens RBcDNA including the perfect coding array of RB stirp was introduced into the AcNPV expression vector, and the recombination virus was bred in the insect cell. The Homo sapiens pp110RB manifestation was realizable with a high level with the host-vector system. The obtained protein was phosphoryl-ized and the nucleus of an infected cell was correctly made into the target. Furthermore, the purification method of RB protein was also established. In addition, according to this invention, completely like Shinsei Homo sapiens pp110RB, purification protein combined DNA and has formed an SV40 T antigen and specific complex. Moreover, according to the proteinic nuclear transformation immediately performed after the microinjection, it became clear that this protein showed activity and could use it for a therapy.

[0051] In order to produce RB protein in the configuration Baculoviridae manifestation system of B1. recombination Baculoviridae to the maximum extent, it rearranged by removing a great portion of 5' non-coding

array from RB gene, and the transition vector was constituted. By seat singularity mutagenesis, two BamH1 seats were introduced into NUKURECHIODO 116 and 2935 of RBcDAN, and the configuration of a recombination transition vector was promoted. PORIHE drine compounds 5' a non-coding array with refined pAcYM1/RB2.8 perfect (60 base pairs) as shown in drawing 1] -- 5' of RBcDNA -- it unites with a non-translated field and mRAN which a perfect coding array follows after that is coded. This recombination gene does not contain an ATG codon for the upstream of NUKURECHIODO 139 of Shinsei RB initiation seat. Therefore, a recombination gene codes un-uniting and full length RB protein.

[0052] Although the transition vector pAcYM1 is shown in drawing 1 , these all have the upper array of the PORIHE drine compounds gene containing A of an initiation ATG codon, and BaMH1 seat characteristic next continues. There is a publication of Matsuura etc. about a transition vector. J. Gen.Virol., 68:1233-1250 (1987). pRB 44-2 includes the perfect RBcDNA coding array which results in the nucleotides 116-2935 which carried out sub cloning in BamH1 seat of the plasma pGEM1 (Promega). About the recombination Baculoviridae vector, pAcYM1/RB2.8 [i.e.,], since BamH1 fragmentation of 2.8kb was inserted and constituted from pRB 44-2 in the proper direction of orientation at BamH1 seat of pAcYM1, the imprint of Lycium chinense of RB gene was completed in the bottom of direct control of a polyhedrin promotor.

[0053] The following points were taken into consideration, when a Baculoviridae manifestation vector was constituted and pp110RB was compounded. pRB 44-2 becomes BamH1 seat of pGEM1 from the perfect RBcDNA coding array which results in the nucleotides 116-2935 which carried out sub cloning. Including EcoR1 fragmentation of about 7 kbs of a virus DNA array located in the side face of a PORIHE drine compounds gene, in this case, pAcYM1 permutes all the PORIHE drine compounds coding arrays except the 1st A of ATG by BamH1 linker, although the leader sequence remains as it is. Since BamH1 fragmentation of 2.8kb was inserted and constituted from pRB 44-2 in the proper direction of orientation at BamH1 seat of pAcYM1, the imprint of Lycium chinense of RB gene was completed about a recombination Baculoviridae vector including polyhedrin promotor RBcDNA fusion, pAcYM1/RB2.8 [i.e.,], in the bottom of direct control of a polyhedrin promotor. The array of a fusion joint is shown in the direction under drawing 1 . Moreover, the case notation under drawing 1 shows a polyhedrin promotor, and the upper case notation shows a RBcDNA array. And BamH1 linker is underlined. An arrow head shows the translation of the fusion gene using ATG (nucleotide 139) of RB. a* (+1) of drawing 1 shows the 1st A of the translation initiation codon ATG of a PORIHE drine compounds gene.

[0054] The imprint to a viral genome from the recombination plasmid of RBcDNA is a wild type Autographa by the RIPOFE cushion (lipofection). It carried out by carrying out the conte run SUFE cushion (contransfection) of the PaCym1/RB2.8DNA using a californica nuclear polyhedrosis virus DNA (BRL). Since a PORIHE drine compounds closing object was not accepted in an infected cell, the recombination virus which inactivated the PORIHE drine compounds gene by the ARERIKKU permutation with RB gene by family recombination was identified according to the plaque gestalt. Plaque purification of the virus was carried out 3 times, and the pure Baculoviridae stock was obtained. This was set to AcNPV-Y4RB.

[0055] Before the manifestation AcNPV polyhedrin promotor of the exogenous RB protein in B-2. infection insect cell determines whether Homo sapiens RB gene expression is brought about as a different-species invertebrate cell Sf9 cell is prepared. The yeast rate of 3.33 gm/l (yeastolate), insect culture-medium (Grace's insect medium)-Bull.1555 (a Texas (1987) agricultural experiment place --) of the grace which added a lactalbumin hydrolysis production object (GIBCO) and 10% of heating inactivation fetal calf serum (GIMINI), A college experiment place and TX are used and it is Sf9, i.e., Spodoptera, by the monolayer or suspension culture at 27 degrees C. frugiperda IPLB-Sf21-AE [In vitro, 13:213-217 (1977)] was cultivated. When carrying out large-scale production of the cell lysate, the spinner culture of Sf9 cell was cultivated by the EX-CELL400 convention culture medium (J. R.Scientific) which does not contain a blood serum. Suspension culture of the malt (Molt) -4 cell of Homo sapiens T leukemia cell lineage was carried out by RPMI1640 culture medium which added the calf serum 20%. Saos-2 cell of osteosarcoma cell lineage was cultivated by the Dulbecco (Dulbecco) denaturation Eagle's medium which added fetal calf serum 7.5%.

[0056] When an AcNPV PORIHE drine compounds prow motor determined whether Homo sapiens RB gene expression is brought about as a different-species invertebrate cell, infection processing of the Sf9 cell was carried out by plaque purification AcNPV-Y4RB. 40 hours after infection, the lysate of an infected cell was collected and immunoprecipitation processing was carried out with anti--RBO-47 antigen. Next, after carrying out SDS-PAGE processing of the sample, western blotting analysis was carried out.

[0057] The pp110RB identification result in the AcNPV-Y4RB infection insect cell performed to drawing 2 and

drawing 3 by western blotting analysis is shown. In the case of drawing 2 , the cell extract was prepared 40 hours after infection from the simulation infected cell (path 2), the AcNPV-Y4RB infected cell (path 3), or wild type AcNPV infection Sf9 cell (path 4). The malt -4 of Homo sapiens leukemia cell lineage was used as contrast (path 1).

[0058] Although the manifestation was accepted in the extract (path 3) of an AcNPV-Y4RB infected cell in RB protein of the same full length as a mammalian cell (path 1) when immunity blot processing was carried out by the monoclone system antibody as shown in drawing 3 , in the case of the simulation infected cell or the wild type AcNPV infected cell (paths 2 and 4), it did not accept. In order to ask for the optimal stage of RB protein production, in the case of drawing 3 , the time amount after infection was changed, and the cell extract was obtained from AcNPV-Y4 infected cell in it. Immunoprecipitation processing of the lysate was carried out with anti--RBO.47 antigen, and immunity blot processing was carried out by the pMG3-245 monoclone system antibody. p110RB of drawing 3 and pp110RB express non-phosphoryl-ized protein and phosphoryl-ized protein, respectively. After infection acted as the monitor of the production of RB protein, and asked for the optimal stage to collect cells. As shown in drawing 3 , RB protein production could be detected 24 hours after infection, and the 12 next hours increased the amount of production considerably. Protein production level was maintained all the time over about 72 hours after infection, and the bacteriolysis of a cell started it substantially 72 hours after. In order to suppress protein degradation accompanying lysis to the minimum, infected cells were usually collected about 40 hours after infection.

[0059] When detecting the manifestation of RB protein, AcNPV-Y4RB was used and infection processing of the Sf9 cell was carried out by MOI of 0.5. The cell of 5x104 was dissolved 24, 36, 48, 60, and 72 hours after from infection in the 1ml dissolution buffer solution (Naf [of Nonidet (Nonidet) P-40;1mM / of EDTA;100mM] of NaCl;50mM of NaCl of 50mM, and 7.4; 0.2% of pH, and PMSF of 1mM), respectively, at-long-intervals alignment processing was carried out for 5 minutes, and the lysate was defecated. Next, the incubation of the lysate was carried out with anti--RBO.47 antigen, and the immune precipitate was separated by SDS-PAGE 7.5%. Then, protein was moved to nitrocellulose paper and it processed with the conventional method. After leaving it in an overnight cut off state and carrying out incubation processing of the nitrocellulose paper with pMG3-245 anti--fRB monoclone system antigen for 3 hours, according to Cell and the publication of 54:275-283 (1988), it processed by the alkaline phosphatase junction goat **-mouse IgG and the chromophoric substrate.

[0060] As for the phosphoryl-ized RB gene after the nuclear normal position of B3. exogenous RB protein, and a translation, molecular weight (Mr) codes the nucleus phosphoprotein of 110,000. In order to investigate whether RB protein produced into the insect cell by Baculoviridae reached the nucleus correctly, 40 hours after infection, anti--RB0.47 antigen was used and immunity dyeing of the AcNPV-Y4RB-infection Sf9 cell was carried out. The normal position between cells of RB protein discovered into the insect cell by immunity dyeing is shown in drawing 4 and drawing 5 . The case of AcNPV-Y4RB-infection Sf9 cell is shown in the case of mock infection Sf9 cell, and drawing 5 at drawing 4 . As shown in drawing 4 and drawing 5 , the infected cell contained the unusually large nucleus. Such a condition is peculiar to the cytopathogenic effect of the Baculoviridae infection. Dyeing was not accepted when the incubation of mock infection or the wild type AcNPV infection Sf9 cell was carried out with anti--RB0.47 antigen (drawing 4). However, dyeing strong only against the nucleus of an AcNPV-Y4RB infected cell was accepted (drawing 5). When SDS-PAGE and Western blotting analyzed the nucleus and cytoplasm extract from AcNPV-Y4RB infection Sf9 cell, it has checked that exogenous RB protein mainly existed in a nucleus part.

[0061] Immunity dyeing was performed at the following process. Also in both mock infection wild type AcNPV infection and AcNPV-Y4 infection the overnight incubation of the Sf9 cell was inoculated and carried out to the chamber slide (Miles Scientific) which carried out coating of the Polly L-lysine (Sigma) 40 hours after infection. Before moving from all to the following process, the phosphoric-acid buffer sodium chloride water solution washed the slide. That is, the interstitial cell was fixed for 20 minutes for 10 minutes using the acetone (-20 degrees C) using the 0.04M phosphate buffer solution of 4% formaldehyde of ****. After carrying out the pre-incubation of the fixed cell for 10 minutes with the PBS solution of 2% of normal goat blood serum, the overnight incubation was carried out with anti--RB0.47 antigen diluted to 0.02% of triton X-100. The BIOCHINIRU-ized goat **-rabbit IgG (Canada and TAGO of Burlingame) was added after washing. 1 hour after, after carrying out the incubation of the interstitial cell for 45 minutes using AB complex joined by the horseradish peroxidase (Canada and Vector Labs of Burlingame), the incubation was carried out using the following substrate. This substrate consisted of 0.05% of 3 and 3'-diaminobenzidine tetrahydro chloride, and 0.01% of a 0.05M tris-HCl solution of H2O2. pH7.6 (Sigma). The

reaction was suspended by carrying out PBS washing of the cell after 3 - 5 minutes. Next, a photograph of a cell was taken with the NIKON diamond photograph microscope (diaphotomicroscope).

[0062] The result of phosphoryl-izing of RB protein produced into the insect cell at drawing 6 and dephosphorylation is shown. 40 hours after AcNPV-Y4RB infection, a 35S-methionine or 32P-orthophosphoric acid was used, and metabolic turnover labeling of the Sf9 cell was carried out in 3 hours. Malt -4 was used as contrast and immunoprecipitation of the cell lysate was carried out using anti--RB0.47 antigen. before carrying out potato acid phosphatase (PAP) processing, (paths 1, 2, 3, and 4) were separated, SDS-PAGE (path 1', 2', 3', and 4 -- ') separated 35S- and a 32P-labeling RB protein immune complex after processing, and radioautography analyzed. Similarly, the dephosphorylation experiment was conducted using the lysate from a non-labeled cell, and western blotting analysis was performed before and after potato acid phosphatase processing (paths 5 and 6 and 5', 6').

[0063] Furthermore, the case of drawing 4 is explained continuously. Phosphoryl-ization of RB protein is produced in a multiplex serine and threonine residue, and the molecular weight heterogeneity of RB protein in SDS-PAGE is explained. In order to investigate whether phosphoryl-ization is received after RB protein produced into [Oncogene Res., 1:205-214(1989);Cell, 56:57-65 (1989)] insect cell translating, a 35S-methionine or 32P-orthophosphoric acid was used from 3 hours after infection in 40 hours, and metabolic turnover labeling of the AcNPV-Y4RB-infection Sf9 cell was carried out. Immunoprecipitation processing of the cell extract was carried out, and radioautography processing was carried out after carrying out SDS-PAGE analysis. What is necessary is just to refer to the paths 2 and 4 of drawing 6 about this point, respectively. It was parallel to this, the immunoprecipitation nature RB protein from the same extract was processed by potato acid phosphatase (PAP), and the dephosphorylation effectiveness RB protein migratory [in SDS-PAGE] was tested. The molecular weight 110,000 of 35S-labeling RB protein fell from the double band to 1-fold band after dephosphorylation (path 2' of drawing 6). And activity emitted almost completely from 32P-labeling RB protein (path 4' of drawing 6). The band change pattern as PAP processing also with the same dephosphorylation analysis by Western blotting about the lysate from a non-labeled cell which carried out infection processing by AcNPV-Y4RB was shown (the path 6 of drawing 6 , 6'). These observation result showed having phosphoryl-ized RB protein produced into the insect cell. Moreover, the molecular weight heterogeneity of RB protein accepted by SDS-PAGE from this change has been explained.

[0064] Radiation labeling and dephosphorylation analysis of Sf9 insect cell were carried out at the process shown below. 40 hours after infection, the incubation of the Sf9 during 30 minutes cell (3x106) was carried out using the 30mm pan by the methionine and the DME culture medium containing neither of a phosphoric acid which added 10% of fetal calf serum. Then, metabolic turnover labeling of the cell was carried out by adding a 0.25 mCi(s)/a ml 35S-methionine (1134 Ci/mmole, NEN) or 0.25 mCi(s)/ml 32P-orthophosphoric acid (support nothing, ICN) in 3 hours. The cell was extracted using the dissolution buffer solution (tris of 50mM - NaF [of Nonidet P-40;1mM / of EDTA;100mM] of NaCl;50mM of HCl and 7.4; 0.2% of pH, and PMSF of 1mM), and immunoprecipitation processing of the extract was carried out with anti--RB0.47 antigen.

[0065] Potato acid phosphatase (PAP and Boehringer) dephosphorylation analysis of two thirds of the immunoprecipitation RB protein from 35S- or the 32P-labeling cell lysate, and the non-labeled cell lysate was carried out. [Oncogene Res., 1:205-214 (1989)]. The incubation of the RB protein content immune complex was carried out using PAP of 1.5 units in 37 degrees C (leupeptin of MES of 20mM, and 2; 50micro [of NaCl;1MgCl(s) of mM of pH5.5;100mM] M) of reaction buffer solutions, and 60 minutes, and after the reaction, after carrying out SDS-PAGE analysis 7.5%, radioautography or Western blotting analyzed RB protein.

[0066] Infection processing of the Sf9 cell was carried out by AcNPV-Y4RB by the multiplicity of infection (MOI) of purification 1.0 of RB protein from B4. infection insect cell, and the cell lysate was obtained 40 hours after infection. The total level of RB protein discovered in the Baculoviridae system on this condition was about 17-18mg per 1l. of infection insect cell culture objects ("109 cell). About this point, **** which illuminates Table 1 3 is good.

[0067]

[Table 1]

Table [ ] 1 purification process of the recombination RB protein from the Baculoviridae infection insect cell Sum total tongue RB tongue Yield Whenever [purification] Purity Park mass Park mass (%) (%)
(mg) (mg) A cell extract 670a 16c 90c 1.0 times 2.3pMG3-245 immunity affinity column 13.5b 12.9d 72 cds 41.3 times 95 Protein quantum c. western blotting by the protein quantum b. micro BCA (PIERCE) by the approach and spectrophotometry of a. Bradford (Bradford) (Bio-RAD) And the protein quantum by the protein quantum d.

Coomassie-brilliant-blue-staining method by the density measurement method, and the density measurement method [0068] As shown in Table 1, 90% of discovered RB protein (16mg) was accepted in the supernatant after cell division, and 10% had stopped at the insoluble fraction. Since 2.3% of all cell protein was formed, RB protein was easily detectable from the cell lysate. About 135mg protein was recoverable from the alkaline eluate of a column after one-step immunity affinity chromatography purification. In order to investigate the purity of elution RB protein, SDS-PAGE and Coomassie brilliant blue staining analyzed the eluate aliquot equivalent to 2.5x105 cell.

[0069] What is necessary is just to refer to drawing 7 which shows immunity affinity chromatography purification of pp110RB about this point. After analyzing the aliquot (equivalent to 2.5x105 infected cell) of eluate with the electrophoresis method by 10%SDS-poly AKURIRO amide gel from the rough lysate from mock infection Sf9 cell (path 1) or AcNPV-Y4RB infection Sf9 cell (path 2) of 1x105, and pMG3-245 anti--RB immunity affinity chromatography, it analyzed by the Coomassie-brilliant-blue-staining method. The arrow head has shown RB protein of anticipation molecular weight.
[0070] When judged by the density measurement method, the above-mentioned one-step purification process is effective, and RB protein was able to be obtained by 41.3 times whenever [95% / of purity / (drawing 7 , path 3), 72% / of yield /, and purification] (Table 1).
[0071] Some modification was added although the immunity affinity column was constituted according to the publication of Shneider, etc. ****, etc. [J. Biol.Chem., 257:10766-10769(1982);Virology, 144:88-100(1985)]. After filling up the Bio-Rad (Bio-Rad) column with 2ml protein G-agarose (Genex) and washing by 0.01-N HCl, the joint buffer solution (sodium acetate of 0.1M, NaCl of pH5.0;0.1M) washed. It joined together by adding the anti--fRB monoclonal antigen (pMG 3-245) of 2mg [15] to a column. Next, the boric acid buffer solution of 0.1M washed the column widely, and the bead was re-suspended in the 20ml buffer solution. Dimethyl PIMERIMI date dihydrochloride (Sigma) was added so that the last concentration might be set to 40mM(s), mixture was stirred at the room temperature for 1 hour, and crosslinking reaction was performed. after washing and a room temperature -- for 10 minutes, 20ml solution of ethanolamine-0.1M boric acid buffer solutions of HCl of 40mM, and pH8.0 -- it came out and the residual reactivity radical of a bead was intercepted. then, the glycine of 0.2M and pH2.3 -- it came out and the column was washed, and it saved in this solution until it neutralized and was needed with tris buffers (tris-HCl of 50mM, EDTA [of pH7.4;100mM] of NaCl;1mM of PMSF:1mM). When OD280 of the first monoclonal antigen sample and OD280 of the flow fraction in the following process were measured, about 10mg pMG 3-245 had combined with the protein G-agarose which is 2ml.
[0072] About RB protein, two biochemical properties are described despite specific complex formation current [with B5.DNA avidity and an SV40 T antigen]. That is, it is one of them that DNA is combinable as a peculiar property, and it is one more [Nature, 329:642-645 (1987)] and that how many kinds of the neoplasm protein and the specific complex of that DNA tumor virus can be formed [Cell, 54:275-283,;(1988) Science, 243:934-937 (1989);Nature (London), 334:124-129 (1988)]. About these [which are participating in proteinic biological functions] two well-known biochemical properties, RB protein refined from the Baculoviridae infection insect cell was tested.
[0073] A south Western DNA-joint measuring method is shown in drawing 8 and drawing 9 . The purification trpE-RB fusion protein of 6microg and purification Baculoviridae manifestation pp110RB were applied to SDS-PAGE 10%. In the case of the joint measuring method shown in drawing 8 , the Coomassie-brilliant-blue-staining method was used, and, in the case of the joint measuring method shown in drawing 9 , the electric imprint of the parallel gel was carried out at nitrocellulose paper. Next, the incubation of the blot was carried out by the 32P-labeling DNA fraction, and radioautography analysis was carried out. In the case of drawing 8 and drawing 9 , it is as follows. Path 1:RB19-22; path: -- RB23-27; -- path 3:RB19 -- the purification RB protein from a -27; path 4:AcNPV-Y4RB infection insect cell.
[0074] In the case of drawing 8 and drawing 9 , DNA binding was measured by south Western analysis. In this case, SDS-PAGE separated the trpE-RB fusion protein of tales doses, and the purification RB protein from an insect cell 10%. The amount of the protein by which loading was carried out was calculated by the Coomassie-brilliant-blue-staining method (drawing 8). In the experiment which used another parallel gel, after carrying out the electric imprint, the incubation was carried out to the nitrocellulose membrane by the 32P-labeling DNA. Radioautography analyzed next DNA combined with protein (drawing 9). The affinity to DNA of the fusion protein RB 19-27 with which the main domain interacts with DNA is 20 times as high as two fields RB 19-22 and RB 23-27. About this point, the path 3 of drawing 9 could be compared with paths 1 and 2, and purification full length RB

protein showed strong DNA avidity like RB 19-27 (drawing 9 , path 4). The DNA avidity of the purification RB protein from an insect cell is elution which continues after that from a column even if based on maintenance of the protein by the DNA cellulose. - It has proved also by NaCl- of about 400 mM(s).
[0075] Purification of pp100RB from an infection insect cell was carried out as follows. By MOI of 1.0, using AcNPV-Y4RB, infection processing was carried out and suspension culture of the Sf9 cell was carried out (1X166 cell / ml, 1000ml). 40 hours after infection, low-speed centrifugal separation pelletizes and washes a cell, and it is tris of 50mM(s). - It re-suspended in the extract buffer solution containing NaCl;10% the leupeptin of PMSF;25microg of DTT;1mM and 50 units of NP-40;1mM of glycerol (v/v);1mM of HCl and 7.4; 0.2% of pH / aprotinin of ml. [of EDTA;100mM] After carrying out an incubation for 15 minutes in Hikami, centrifugal (for 10,000xg, 4-degree-C, and 10 minutes) clarification processing of the sample was carried out, and RB content supernatants were collected. As mentioned above, immunity affinity chromatography of pp110RB was carried out in the column of 2ml capacity containing the anti--fRB monoclonal antigen (pMG 3-245) combined with protein G-agarose. After letting a supernatant pass 4 times in a column, the following solution was used in the amount of 200 bed capacity, respectively, and the column was washed continuously. The dissolution buffer solution, the dissolution buffer solution containing NaCl of 500mM, and a penetrant remover (PMSF;10% glycerol [of 200mM / of NaCl;1mM] of EDTA;1mM of DTT;1mM). Next, binding protein was eluted from the column with the alkaline elution buffer solution containing PMSF of EDTA;1mM and 10% of glycerol of NaCl;1mM of the triethylamine of 2mM, and pH10.8;200mM. 1ml fractions are collected and it is tris of 1M immediately. - It neutralized by 1/12 capacity of HCl (pH7.5), and saved at -70 degrees C in 10% glycerol.
[0076] When refining pp110RB from an infection insect cell, after calculating the total amount of protein, the south Western DNA binding measuring method and the SV40 T antigen joint measuring method were enforced.
[0077] The amount of all the protein of the elution fraction of an immunity affinity column was calculated with the micro-BCA measuring method (PIERCE). SDS-PAGE analyzed the elution protein sample next, and the Coomassie-brilliant-blue-staining method and the degree were asked for the amount of RB protein of eluate by the density measurement method. The total amount of protein of a cell extract was measured by the approach (Bio-Rad) of Bradford. [Anal.Biochem., 72:248-254 (1976)]. In order to carry out the quantum of the RB protein of the cell lysate, after using the purification RB protein diluted in order as a criterion and carrying out Western blotting, density measurement compared band strength. What is necessary is just to refer to Table 1 about this point.
[0078] Protein blotting was carried out with the conventional method. According to Bowen's (Bowen) etc. publication, the incubation of the blot was carried out by the radiation labeling DNA. [Nuleic Acids Res.8:1-21 (1980)]. This was performed at the room temperature. After rinsing the blot quickly, it washed 3 times by the urea of 6M, and 0.2% of NP-40 (all are 20 minutes), and washing (all are 30 minutes) was succeedingly carried out 4 times with the DNA binding buffer solution (NaCl;0.2% BSA;0.2% Ficoll400 and 0.2% of polyvinyl pyrroline of EDTA;50mM of tris-HCl of 10mM, and pH7.0;1mM). Next, the incubation of the blot was carried out for 30 minutes in the DNA binding buffer solution including the 32P-labeling DNA. By EcoR1, line-ized pGEM1DNA was labeled by the alpha-32P deoxy nucleotide (Amersham, >3000 Ci/mmol) by the random priming, and it was used as a probe. Washing of the blot was carried out 3 times (all are 10 minutes) with the DNA binding buffer solution after hybridization, it was air-dry and radioautography analyzed. TrpE-RB fusion protein was included as contrast. Each trpE-RB fusion protein was named according to the exon of RB gene which protein contains. That is, RB 19-22, RB 23-27, and RB 19-27 occupy the pp110RB field of an exon 19-22 (amino acid 612-775), an exon 23-27 (amino acid 776-928), and an exon 19-27 (amino acid 612-928), respectively.
[0079] Immunity chromatography purification of the SV40 T antigen was carried out from Ad-SVX1-infection 293 cell. [J. Virol., 53:1001-1004(1985);Cold Spring Harbor Press., Cold SpringHarbor, NY, pp.187-192(1982)]. And anti--T monoclonal PAG419 antigen was obtained from Oncogene. The well-known complex formation measuring method was enforced. However, some modification was added. That is, the Baculoviridae manifestation RB protein of 800ng was mixed with the 1ml EBD buffer solution (NaCl of tris-HCl of 50mM, and pH8.0;120mM, and 0.5% of Nonidet P-40) containing PMSF of 1mM, 25microg [/ml] leupeptin and 50 units / aprotinin of ml. The purification T of 800ng(s) was added to mixture, and the incubation was carried out in Hikami during 90 minutes. anti- -- using either of -RB0.47 or PAB419 antigens, immunoprecipitation of the aliquot of mixture was carried out and Western-blotting analysis was carried out. The blot made it react with pMG 3-245 and PAB419 in order. After carrying out an incubation with the alkaline phosphatase junction goat **-mouse IgG, coloring processing of the blot was carried out using the color-enhancing substrate.

[0080] In order to test the capacity for purification RB protein to be able to form an SV40 T antigen and specific complex, equivalent RB protein and an equivalent T antigen were mixed, and immunoprecipitation of the aliquot of mixture was carried out using anti--RB0.47 antibody or -anti-T antigen PAB419.
[0081] In relation to this point, the complex which Baculoviridae manifestation RB protein and an SV40 T antigen form in drawing 10 is shown. Purification Baculoviridae manifestation RB protein was mixed with the purification T antigen by in vitro one. next, the mixture of this aliquot -- PAB419 (path 2) -- anti- -- immunoprecipitation was carried out by either of -RB(s)0.47 (path 3), and Western blotting analyzed. Paths 1 and 4 show the purification Baculoviridae manifestation RB protein which carried out immunoprecipitation by the purification SV40 T antigen which carried out immunoprecipitation by PAB419, respectively, and anti--RB0.47 antibody.
[0082] As shown in drawing 10 , when the T antigen was mixed with RB protein by in vitro one, PAB419 not only coimmunoprecipitates with RB protein, but (the path 2), and T and anti--RB0.47 antigen coimmunoprecipitated (path 3). It is proved [data / these] that RB protein from the Baculoviridae infection insect cell can form an SV40 T antigen and specific complex.
[0083] Since it became clear that the nuclear transformation purification protein of B6. purification RB protein had the two well-known biochemical properties of RB by in vitro one, it investigated about the in vitro behavior of purification protein next. The cytoplasm of Saos-2 cell with the osteosarcoma cell lineage without an exon 21-27 which codes C terminal cutting RB protein (p95), including a deficit RB gene was injected with purification RB protein. [Proc.Natl.Acad.Sci.U.S.A., 87:6-10 (1990)]. The antibody orientated this protein to the cytoplasm in consideration of the fact of going to the C terminal of RB protein, in the small quantity of extent which is not accepted by anti--RB0.47 antibody to be used. Immediately after injection, the cell was fixed and it analyzed by the immunity staining technique. The nuclear normal position of generation RB protein after carrying out a microinjection to drawing 11 to the cytoplasm of Saos-2 cell is shown. The cell was injected with generation RB protein and it analyzed by the immunity staining technique. The arrow head shows among drawing that dyeing of the nucleus after a microinjection is strong compared with the non-injecting cell.
[0084] As shown in drawing 11 , compared with a non-injecting cell, dyeing of the nucleus after a microinjection is strong (arrow head), and it is shown that injection protein was immediately conveyed to the nucleus. since RB protein is known as a nucleoprotein -- the nuclear normal position of after a microinjection and purification protein -- immediately -- and although cut correctly, this has suggested that this protein is activity in in vivo one.
[0085] In the case of the microinjection, purification RB protein was dialyzed so that the last concentration might become [ml] the injection buffer solution containing DTT of EDTA;0.1mM and 2% of glycerol of KCl;0.1mM of tris-HCl of 20mM, and pH7.4;10mM in 0.5mg /. The capillary glass tube needle (Eppendorf) was used and the microinjection of the Saos-2 cell which grew on the glass CHANN bar slide was carried out by the usual approach. The micro manipulator and opposition contrast microscope (NIKON make) of Eppendorf which attached the vacuum pressurizer were used, and micromanipulation of the capillary tube was carried out, respectively, and the microinjection process was visualized. After the microinjection, the cell was immediately fixed with the 0.04M phosphate buffer solution solution (pH7.4) of formaldehyde 4%, and it analyzed by the immunity staining technique.
[0086] As explained more than B6. epitome, it has proved that the bottom Homo sapiens retinoblastoma gene production object of the transcriptional control of the Baculoviridae polyhedrin promotor could be efficiently discovered. Since RB protein had the misgiving which it not only blocks growth of a cell, but shows "toxicity" to it depending on the case, it was thought that the attempt which has discovered RB protein on high level for a long time was difficult. The imprint of the foreign gene from a polyhedrin promotor is produced in the second half of infection, and cutoff of production of an extracellular virus particle, a cell, and a great portion of virogene continues next. Therefore, when superfluous production is carried out, the Baculoviridae insect cell lineage is advantageous to composition of protein, such as RB protein which has that it is harmful to cell growth. Another advantage of this system is in the similarity of an insect and the protein synthesis path of a mammalian cell.
[0087] Moreover, it also becomes clear that RB protein reaches the nucleus of an insect cell correctly, and this connotes that the nuclear normal position signal of mammalian is recognized by the insect cell. Although glycosylation of the recombination protein in the Baculoviridae manifestation system is restricted to O-association of a high mannose mold, and an N-joint oligosaccharide, suitable phosphoryl-ization of outpatient department protein is reported about c-myc and the manifestation of HTLV-Ip40x (J. Virol.). Although RB protein was phosphoryl-ized from before, not carrying out glycosylation was known. This is a reason for production of functional RB protein with the suitable Baculoviridae manifestation system.
[0088] As indicated on these specifications, RB protein produced into the infection insect cell is phosphoryl-ized.

after a translation, therefore can specialize a multiplex band by Western-blotting analysis completely like the case of RB protein of Shinsei mammalian. However, when seeing from band strength and it compares with hyper-phosphoryl-ized RB protein, phosphoryl[non-]-izing and a phosphoryl-ized gestalt are superior. Since this phenomenon's being reflection of the cell cycle condition of the population at the time of viral lytic infection or a lot of exogenouses RB exist in a cell the place to current, it is not known whether it is because phosphoryl-izing of the protein by the insect kinase is only inadequate. In order to determine the same thing **** as the case of phosphoryl-ized PATANGA mammalian protein, and accuracy, it is necessary to map the phosphoryl-ized location of RB protein in a precision.

[0089] The total level of the recombination RB protein discovered in the Baculoviridae system is about 17 to 18 mg per 1l. (109 cells) of infection insect cell culture objects. This manifestation level is equivalent to other mammalian protein which this system generates. For example, in the case of interleukin 2, it is 10-20 mg/l [The Banbury Report.Fields, B., Martin, M.A.& Kamley, D. (ed.), 22:319-328 (1985), Cold SpringHarbor Laboratory Press, Cold SpringHarbor]. moreover, [Oncogene, 4:759-766(1989)]. which is 4-5 mg/l in P210 BCR-ABL -- if a recombination transition vector including the 5 'PORIHE drine compounds 5 without deficit united with RBcDNA which removed most non-coding fields' non-translated field is used, high RB protein manifestation level can be made still higher. This RBmRNA array is G+C Rich highly, and is a factor advantageous to forming stable secondary structure. These structures are considered to make translation effectiveness of Correspondence mRNA low when it is before an initiation codon. about the in vitro translation of RBmRNA, it proves [become / 10 times / high] from 5 times by permuting RB5' a non-translated array by it of an alfalfa mosaic virus (AMV) RNA 4 or beta globulin mRNA -- having -- this -- RB5' -- [EMBO J. (1990)] which is what suggests having a bad influence on the translation of a non-coding array potentially. Moreover, if 5' non-translated array with a long foreign gene exists, it will also have become clear that the recombination PORIHE drine compounds manifestation in the Baculoviridae system is influenced. since A+T rich nature of a polyhedrin promotor is very strong, before inserting in a transition vector as a conclusion -- long -- and G+C -- rich 5' non-coding array should be trimmed from RBcDNA, and the pp110RB manifestation should be optimized.

[0090] In order to suppress the denaturation of the protein under purification to the minimum, several kinds of different protocols were tested about elution of RB protein from an affinity column. Since many are not known so much about the biological functions and the biochemical property of RB protein, it is only two parameters that it can be used as a scale of the integrity of purification protein. That is, it is complex formation with DNA avidity and an SV40 T antigen. In addition, the conditions which maintain a proteinic biochemical property were elution conditions which use the triethylamine of 20mM(s) by pH10.8 in this invention. Although purification protein carried out the nuclear normal position immediately from the cytoplasm after the microinjection, it is proved [this / protein / this] that activity is shown under these elution conditions. When protein was eluted by extreme pH (triethylamine of the glycine of 200mM, pH 2 and 3, or 100mM(s), pH11.5), the inclination for protein to denaturalize was accepted. That is, the two above-mentioned activity fell. This is clear also from insoluble floc having formed after prolonged preservation.

[0091] According to the former report, although [non-phospho RURIRU-ized RB protein] the SV40 T antigen of D2C2 cell which is the stable transformant by the SV40 T antigen of nephrocyte network valve flow coefficient1-P of an ape is combinable, it was found out that the HIPOHOSUHORIRU-ized gestalt of [Cell, 56:57-65 (1989)] and RB protein of a certain kind can also form an SV40 T antigen and complex. This has been checked with sufficient repeatability, when in vitro mixing of the purification RB protein [from a T antigen and an AcNPV-Y4RB infection insect cell] or malt -4 lysate was carried out. The same phenomenon was accepted also when complex was formed by in vivo one using a Cos cell (drawing 10). Since phosphoryl-ization of RB protein is changed in a cell cycle in the case of phase singularity and the complex formation between RB and viral neoplasm protein is participating in the transformation activity of these DNA tumor virus, the relevance of HIPOHOSUHORIRU-ized RB protein and an SV40 T antigen should be solved someday.

[0092] RB protein which does not have a meltable deficit when the Baculoviridae insect cell lineage is used, as explained above and which can also predict activity suddenly -- an owner -- it is a big advance for future biochemical and research of a biophysical property of RB gene production object that it can be used in a meaningless amount. They are analysis of the cell protein in relation to the case considered, three-dimension-structure research of RB protein using separation and X-ray crystallography of the specific DNA array with which these interact, etc. Moreover, the elucidation of the biological functions of the retinoblastoma gene in cancer control should also progress. About the intervention to the cell growth of RB and differentiation which were

directly tested by the microinjection considered, it is inquiring on the current energy target.
[0093] The notation used on these specifications is explained below. cDNA -- complementary DNA and kd -- KIRODARUTON and kb -- a kilobase and SDS -- for Nonidet P-40 and MES, sodium salt (2-[N-morpholino] ethane sulfonic acid) and MOI are [a sodium dodecyl sulfate and PAGE / polyacrylamide gel electrophoresis and NP-40 / relative molecular weight and PAP of the multiplicity of infection and Mr] potato acid phosphatase. The protein production object shown by "pp110RB" is the same protein production object as "ppRB110."
[0094] It is included by the pneuma and the range which various kinds of modification is possible and all indicated to the claim although this invention has been explained about the specific example. That is, this indication does not mean a limit.

[Translation done.]

TECHNICAL FIELD

[Industrial Application] On the whole, this invention relates to the cell therapy for treating the production approach of a gene protein production object, and a cell, and controlling tumorigenesis. This invention relates to the cell therapy method which controls a cell therapy and tumorigenesis again. This invention is government support (license number EY05758) invention, and is invention attained in collaboration with national American Insurance Association (the National Institute of Health) and a cull FORUNIA university. Therefore, about this invention, the government also has a fixed right.
[0002]

[Translation done.]

TECHNICAL PROBLEM

[Description of the Prior Art] There is research quite detailed about the tissue of Homo sapiens or an animal, and much deficits are checked on cell level. Many of these deficits have a hereditary cause, and most depends it on a deficit gene. A deficit may consider point mutation etc. as a cause, and as a result, the nucleotide sequence of the gene itself may divide, or it may show an unusual change. When there is a malfunction gene, the gene protein production object with which it becomes impossible to have produced the gene protein production object finally, and itself suffered a loss will be produced.

[0003] A gene therapy can be applied when identifying a deficit gene about both Homo sapiens and an animal. In this case, what is necessary is to lead to the nucleus of the cell which should treat a cloning gene, and just to correct an unusual genetic material or a deficit genetic material. The matter used for this approach has a high manufacturing cost, and needs a complicated elaborate experimental device, and needs application of a complicated elaborate genetic manipulation technique. Generally such a technique cannot be used, therefore only the laboratory where most treats molecule genetic manipulation comparatively elaborate to a small number of altitude can be used.

[0004] Costs of a gene therapy are high, and since it generally cannot use, another cell therapy is searched for. It is possible to draw a conclusion from analysis of the molecular structure and the function of a protein production object about the insurance of the gene which produces this production object. For example, what is necessary is to use a gene protein production object in many cases, and just to opt for the interaction between genes, in order to determine the neoplasm control mechanism of the body. What is necessary is just to refer to the parent-patent application specification of this application about a neoplasm control mechanism. the top where it is reliable and cost is low in order to solve a gene function and a gene interaction -- dependability -- the approach of producing the gene protein production object which can offer a lot of [possible / prediction] high protein is very desirable.

[0005] Not only a gene protein production object is useful, but [although a gene function and a gene interaction are solved] this protein itself can use it for the therapy of a deficit gene condition. In this case, it is advantageous to introduce the suitable gene protein production object for a cell with a deficit genetic material, and it is effective. Cost is low and sometimes easier than the case where the direction of installation of a protein production object prescribes the genetic material itself for the patient for the purpose of a therapy by the case.

[0006] What is necessary is just to refer to the patent application specification submitted to this application and coincidence about a protein therapy.

[0007] When it sees from the recognition about the importance of a gene protein production object, it is very advantageous that the approach of preparing and separating a gene protein production object in the form refined substantially can be used now. in order that that the activity matter can be used as it is so much biochemically may study the biochemical property and molecule behavior which are participating in the heredity mechanism -- a therapy sake -- an owner -- it is a meaningless advance.

[0008] Generally, in production of a different laboratory scale from the case of extensive production, the gene protein production object is produced also by the synthetic production only from the cell. Speaking of the induction from a cell, little existence of the cell protein is recognized very much. Consequently, the attempt which guides sufficiently a lot of protein from the source of nature is not realistic.

[0009] Speaking of the synthetic producing method, the attempt which introduces the decode array of a gene into a bacteria expression vector, and discovers protein was not successful except for some cases. Bacteria production protein has weak solubility. The point of giving the result which a bacterial cell could not change eukaryote protein, and analysis of such protein mistook when modification after a translation was required about protein has another fault which uses a bacteria manifestation system. That is, generally bacteria production

protein has low solubility, and since there is a molecule deficit, usefulness has a limit.
[0010] It not only cannot produce enough a lot of protein, but by the usual laboratory-approach which makes a protein production object, the purity of production protein is not enough, either and variation is in purity. TrpE-RB fusion protein may be discovered for the example of representation of the difficulty at the time of producing a certain kind of protein production object, T7 RNA polymerase discovered to E.coli may be used for it, and a polypeptide may be produced. These approaches are comparatively complicated and need complicated elaborate biochemical actuation. Furthermore, since the target polypeptide does not carry out little deer production very much, these approaches have a big limit. Moreover, the polypeptide produced by this approach is seen in molecule, and it is common not to be desirable. For example, it is because this is not phosphoryl-ized.
[0011] Therefore, biochemical [desired] and the desired approach of producing a polypeptide with a biophysical property in a substantial amount are strongly desired from the importance of a gene production object polypeptide.

[0012] A remarkable advance is seen about an understanding of the function of the gene in living body health maintenance. If that it can generally say cannot produce protein with proper cytogene, various kinds of abnormalities will occur to a living body. It can ask that the whole gene is lost or the gene itself is missing by various kinds of causes for this reason that cannot be produced. From recognition of these factors, remarkable progress is accepted in a gene therapy.
[0013] For example, it is thought that the Wilms' tumor which is infantile kidney cancer is produced by gene inactivation of a chromosome 11. It is proved that oncogenicity can be controlled, if the normal chromosome 11 is introduced into a Wilms' tumor cell when the minute cell fusion medium transferring method of a single strand chromosome is used. On the other hand, this effectiveness is not accepted in Chromosome X and installation of 13.
[0014] On experiment level, although a certain extent of transition of a human chromosome without a deficit is useful, such transition is not applicable to the therapy of a genetic defect. Preparation of the suitable chromosome for a therapy not only needs remarkable skill, but requires time amount for one upwards, and it is because cost is high. For this reason, in many cases, this approach is nonpermissible.
[0015] When it is not desirable to use a chromosome without a deficit for a therapy, thinking theoretically below is introducing into a patient the proper whole gene or the part which has the effect at least. Although this approach has workability higher than the approach of introducing a chromosome without a deficit, it is only a certain fixed case that a gene therapy is desired.
[0016] Not only cost is very high, but separation of the proper nucleic-acid matter, sequencing, and cloning require time amount about this point. In addition, the complicated elaborate molecule genetic manipulation approach which can be used only in the location restricted very much also in the world is required of such an approach. Furthermore, such an approach produces a lot of matter suitable for a therapy even now.
[0017] As mentioned above, a specific therapy of the cell level which cost is comparatively low, and it is reliable, and can more generally use by using the bionics-approach, and uses the specific matter in a biochemical operation is desired strongly. Furthermore, a production object effective in a therapy is introduced on cell level, and a therapy which brings about change of neoplasm control etc. is also desired strongly. Although it is needless to say, it is strongly wished also about the production object which can produce so much in the state of purification without variation, and can be introduced into a deficit cell easily and effectively.
[0018] It is in the 1st purpose of this invention offering a specific therapy safe on the whole and and a production object effective in controlling cancer control.
[0019] It is in another purpose of this invention offering a cancer inhibitory-control production object and a therapy effective in the control and extermination using the bionics-approach and a production object of a cancer tumor. Moreover, it is also the purpose of this invention to offer the drugs constituent for cancer treatment which demonstrates an operation on cell level and the level between cells.
[0020] It is in still more nearly another purpose of this invention offering the drugs constituent which treats the symptom produced by the absence of the deficit or mutation antioncogene which makes nature or a synthetic production object an active ingredient, and an antioncogene.
[0021] This invention leads a specific antioncogene protein production object to an object cell, and consists of a cell therapy which realizes cancer control.
[0022] This invention offers the conventional radiotherapy and the cure for cancer with little need for a chemotherapy. Furthermore, this invention approach can be applied after discovery of disposition which is easy to

suffer from cancer hereditarily at the very early stage before tumorigenesis begins.
[0023] this invention -- an antioncogene protein production object -- good facilities -- and in the point of using it at comparatively low cost and realizing cancer control on cell level, it is very advantageous.
[0024] Although there is a point which has not been clearly carried out about whether cancer will be generated enough when the antioncogene beyond one or it in a certain cell inactivates then, cell installation of a gene protein production object is new approach, and is the advantageous approach to a malignant tumor therapy. This invention is in the condition which suppressed the damage to a living body to the minimum unlike the cytotoxic cancer therapy from the former, and is still more advantageous in the point of bringing about an advantageous change on cell level.
[0025] As mentioned above, it is in the 1st purpose of this invention offering the approach of producing an activity gene production polypeptide without the deficit of a substantial amount.
[0026] Furthermore, another purpose of this invention has structure and a function in offering the same production approach of a specific gene production polypeptide as a natural student polypeptide.
[0027] If it summarizes, the above-mentioned purpose and other purposes of this invention are realizable by the approach of producing a substantial quantity of a request polypeptide by introducing a genetic material into an insect cell. For example, if a cloning gene, gene fragmentation, or its derivative is introduced into a host cell using a suitable vector, it is a substantial amount and the protein of a high grade can be produced with a high level.
[0028] This invention produces the polypeptide of high quality in a substantial amount, offers the approach of carrying out things of investigating a gene function on cell level, and is very advantageous.
[0029] Moreover, this invention is advantageous, is reliable, moreover, can produce this polypeptide at repetitive comparatively low cost, and is very advantageous also in this point.
[0030] Furthermore, this invention can produce the high quality polypeptide of a considerable amount which can solve an intergenic interaction on cell level and the level between cells, and is advantageous also at this point.
[0031]
[Easy explanation of a drawing]
Other purpose, descriptions, and these implementation approaches should become clear from the following publications at the above-mentioned above-mentioned purpose of this invention and the description, and the list. Moreover, it refers to an understanding of this invention itself at an accompanying drawing, and it should be obtained if the publication about the example of invention is read. Drawing 1 is the schematic diagram showing the configuration of the Baculoviridae manifestation vector which compounds pp110RB. Drawing 2 is the western blotting (Western blot) of a ppRB infection insect cell. Drawing 3 is western blotting which shows the cell extract extracted from the infected cell even 72 hours after infection. Drawing 4 is a microphotography in which the normal position between cells of RB protein is shown. Drawing 5 is the microphotography of infection Sf9 cell. Drawing 6 is a radioautograph which shows the result of phosphoryl-izing of RB protein in an insect cell, and dephosphorylation analysis. drawing 7 -- pMG 3-245 -- anti- -- it is drawing showing the rough lysate from -RB, infection Sf9 cell, and the electrophoresis analysis result of eluate. Drawing 8 shows the fusion protein and the south Western (Southwestern) DNA binding measuring method of Baculoviridae-manifestation pp110RB which were applied to SDS-PAGE and a Coomassie-brilliant-blue-staining method 10%. It is the radioautograph of the blot from parallel gel to the gel used in order that drawing 9 might create drawing 8 , and the incubation of this blot is carried out by the 32P-labeling DNA fragment. It is the chromatogram which shows complexing of drawing 10 Baculoviridae-manifestation RB protein and an SV40 T antigen. The nuclear transformation of the purification RB protein after the microinjection to the cytoplasm of drawing 11 Saos-2 cell is shown.

[Translation done.]

EXAMPLE

[The best example of invention] All have adopted the contents here and the reference which makes reference on these specifications constitutes a part of this specification publication. Hereafter, this invention will be explained in the following order.
A. an outline -- the nuclear transformation B7. epitome [0033] of specific complex formation B6. purification RB protein with the RB protein purification B5.DNA-avidity from the phosphoryl-ized B4. infection insect cell after the nuclear normal position of the manifestation B3. extraversio RB protein of the extraversio RB protein in the configuration B-2. infection insect cell of example of B.RB gene production object B1. recombination Baculoviridae, and a translation, and an SV40 T antigen A. When outline this invention introduces a genetic material into an insect cell culture object, it can be used for this gene protein producing a specific gene protein production object by culture about the approach of producing gene protein. If this approach is used, the deficit with a high force value refined substantially can twist, and activity protein can be obtained biochemically.
[0034] The manifestation system of this invention has a large application. That is, for example, Homo sapiens, the cloning gene of an animal, its fragmentation, a homologue, a derivative, or a part is applicable to production of a request protein production object. Thus, needless to say, the produced protein is applicable to the therapy of a deficit cell at the elucidation of a gene function. a flume -- obtaining -- it is because a gene interacts on cell level.

[0035] Since the insect cell culture object had an eukaryote property when a gene protein production object was produced, the suitable thing was found out. In order to introduce a genetic material into a cell culture object, the usual vectors, such as a virus vector, can be used.
[0036] For example, in order to produce RB gene protein production object, the virus vector of an insect cell culture object is used. In order to rearrange with a high level in a culture insect cell and to produce protein about this point, Autographa KARIHORUNIKA of Baculoviridae (Autographa californica), i.e., a nuclear polyhedrosis virus, (AcNPV) is used as a virus expression vector independent of a helper. This virus breeds in the cultivated matrix caterpillar cabbage armyworm from the United States, i.e., a Spodoptera full GIPERUDA (Spodoptera frugiperda) (Sf9) cell. This virus has the promotor with a powerful polyhedron gene adjusted temporarily, and that production object occupies 50% or more of all cell protein at the time of lytic infection. By in vivo recombination, the decode array of a foreign gene receives the translational control of a polyhedrin promotor, and, as a result, discovers protein with a high level. Furthermore, it doubles correctly and the protein which carried out in this way and was produced includes the same modification after a translation as the protein looked at by the original high-class eukaryote.
[0037] This invention is a cell therapy which introduces a specific antioncogene protein production object into an object cell, and realizes neoplasm control. Since a given cell has a deficit gene, in the point which causes the protein manifestation deficit in a cell, it may be missing.
[0038] It is related with this invention approach using the gene protein production object related to a deficit gene. A purification protein production object is introduced into an object cell, for example, neoplasm control is realized. By supporting and introducing this production object into support suitable in pharmacology, this protein production object is cell level, or acts on the bottom level of a cell.
[0039] the cell RB gene protein which whose RB gene which is an antioncogene is missing as an example of this approach, or does not have it was boiled and introduced. What is necessary is just to refer to the parent-patent application specification which has made reference on these specifications about the detailed information about RB gene.
[0040] In the suitable example, the small-child cancer which is a retinoblastoma, i.e., a symptom with the rare

retina under growth, as a PM of recessive tumorigenesis research is used. Based on the normal position and its recessive nature of the heredity element to a chromosome 13q14 which is involving, the gene presumed to be an antioncogene, i.e., a retinoblastoma susceptibility gene, (RB) was cloned. A 4.7 kilobase mRNA imprint is discovered at all in the normal organization which the exon of 27 distributed this gene to the genomic DNA of 200 kilobases, and inspected. By the sequence analysis of a complementary DNA clone, the long open reading frame which has coded the hypothetical protein of the amino acid of 928 became clear. When the antibody to the predetermined epitope guessed from the RBcDAN array was used, as for this RB gene production object, relative molecular weight (Mr) was identified the nucleus phosphoprotein of 110,000-114,000. This was named pp110RB.

[0041] only to a retinoblastoma, it has become clear that the deficit of RB gene function is boiled also at the neoplasm of some of others including a breast cancer, an osteosarcoma, a prostatic cancer, or small cell lung cancer again, and is involving. According to the latest research, if RB gene is reintroduced into a retinoblastoma, an osteosarcoma, or a prostate gland cancer cell by retrovirus medium gene transition, it will have become clear that some fields of new phenotypes including the tumorigenesis in a nude mouse are controlled clearly. This is a direct proof of the neoplasm control function of RB gene. However, the foundation in the molecular level of this biological activity is not established yet.

[0042] Although all the biochemical properties and biological functions of an antioncogene production object are not solved the place to current, it is because there is little this protein in a cell as for this, so it is difficult to obtain in sufficient amount. What is necessary is just to refer to the parent application specification (the name of invention: the production approach of a gene protein production object) for which it applied to this application and coincidence about this point.

[0043] The cell therapy which has a deficit gene or introduces a specific gene protein production object into a cell without a gene was invented. According to this invention, the deficit refined substantially twists and the proper amount of activity gene production protein can be biochemically introduced into a deficit gene with an effective dose on a therapy.

[0044] The cell therapy of this invention has a large application application. This protein production object is useful not only to the therapy of a deficit cell but the elucidation of a gene function. It is because a gene interacts on cell level.

[0045] In the one specific example, a retinoblastoma gene protein production object, i.e., pp110RB, has a deficit gene, or it can apply it to the therapy of an eukaryote cell without a gene widely.

[0046] In addition, according to this invention, completely like Shinsei Homo sapiens pp110RB, purification protein combined DNA and has formed the SV40 T antigen and the specific complex. Moreover, according to the proteinic nuclear transformation immediately performed after the microinjection, it became clear that this protein showed activity and could use it for a therapy.

[0047] B. Explain in more detail about the example of RB gene production, next the production approach by this invention of pp110RB protein. What is necessary is just to refer to the following parent-patent application specifications about information detailed to the pan about this protein and a retinoblastoma gene.

[0048] Although all the biochemical property and biological functions of an antioncogene production object, such as RB gene, are not solved, this is because it is difficult to obtain purification protein in sufficient amount. That is, it is because it is because a cell has little protein and the protein manifestation by the installation to the bacteria expression vector of the coding array of a gene has not succeeded in one except for a part in addition again. It has been concluded that the problem which the approach used now has from these was avoidable if a cloning gene is discovered in an eukaryote system. Although it will be an antioncogene if this invention is illustrated, although the specific example of this invention is related with production of RB gene protein production object, it can apply also to production of the protein production object of other eukaryote genes which are not restricted to this, therefore is related also with production of such a protein production object.

[0049] In order to produce recombination protein with a high level in a culture insect cell, it is desirable although Autographa KARIHORUNIKA of Baculoviridae (Autographa californica), i.e., a nuclear polyhedrosis virus, (AcNPV) is used as a virus expression vector independent of a helper. This virus breeds in the cultivated matrix caterpillar cabbage armyworm from the United States, i.e., a Spodoptera full GIPERUDA (Spodoptera frugiperda) (Sf9) cell. This virus has the promotor with a powerful polyhedron gene adjusted temporarily, and that production object occupies 50% or more of all cell protein at the time of lytic infection. By in vivo recombination, the decode array of a foreign gene receives the translational control of a polyhedrin promotor, and, as a result, discovers protein with a high level. Furthermore, it doubles correctly and the protein which carried out in this way and was produced

includes the same modification after a translation as the protein looked at by the original high-class eukaryote.
[0050] In order to test the manifestation possibility of functional RB protein by the Baculoviridae system, cloning Homo sapiens RBcDNA including the perfect coding array of RB stirp was introduced into the AcNPV expression vector, and the recombination virus was bred in the insect cell. The Homo sapiens pp110RB manifestation was realizable with a high level with the host-vector system. The obtained protein was phosphoryl-ized and the nucleus of an infected cell was correctly made into the target. Furthermore, the purification method of RB protein was also established. In addition, according to this invention, completely like Shinsei Homo sapiens pp110RB, purification protein combined DNA and has formed an SV40 T antigen and specific complex. Moreover, according to the proteinic nuclear transformation immediately performed after the microinjection, it became clear that this protein showed activity and could use it for a therapy.
[0051] In order to produce RB protein in the configuration Baculoviridae manifestation system of B1. recombination Baculoviridae to the maximum extent, it rearranged by removing a great portion of 5' non-coding array from RB gene, and the transition vector was constituted. By seat singularity mutagenesis, two BamH1 seats were introduced into NUKURECHIODO 116 and 2935 of RBcDAN, and the configuration of a recombination transition vector was promoted. PORIHE drine compounds 5' a non-coding array with refined pAcYM1/RB2.8 [perfect (60 base pairs) as shown in drawing 1] -- 5' of RBcDNA -- it unites with a non-translated field and mRAN which a perfect coding array follows after that is coded. This recombination gene does not contain an ATG codon for the upstream of NUKURECHIODO 139 of Shinsei RB initiation seat. Therefore, a recombination gene codes un-uniting and full length RB protein.
[0052] Although the transition vector pAcYM1 is shown in drawing 1 , these all have the upper array of the PORIHE drine compounds gene containing A of an initiation ATG codon, and BaMH1 seat characteristic next continues. There is a publication of Matsuura etc. about a transition vector. J. Gen.Virol., 68:1233-1250 (1987). pRB 44-2 includes the perfect RBcDNA coding array which results in the nucleotides 116-2935 which carried out sub cloning in BamH1 seat of the plasma pGEM1 (Promega). About the recombination Baculoviridae vector, pAcYM1/RB2.8 [i.e.,], since BamH1 fragmentation of 2.8kb was inserted and constituted from pRB 44-2 in the proper direction of orientation at BamH1 seat of pAcYM1, the imprint of Lycium chinense of RB gene was completed in the bottom of direct control of a polyhedrin promotor.
[0053] The following points were taken into consideration, when a Baculoviridae manifestation vector was constituted and pp110RB was compounded. pRB 44-2 becomes BamH1 seat of pGEM1 from the perfect RBcDNA coding array which results in the nucleotides 116-2935 which carried out sub cloning. Including EcoR1 fragmentation of about 7 kbs of a virus DNA array located in the side face of a PORIHE drine compounds gene, in this case, pAcYM1 permutes all the PORIHE drine compounds coding arrays except the 1st A of ATG by BamH1 linker, although the leader sequence remains as it is. Since BamH1 fragmentation of 2.8kb was inserted and constituted from pRB 44-2 in the proper direction of orientation at BamH1 seat of pAcYM1, the imprint of Lycium chinense of RB gene was completed about a recombination Baculoviridae vector including polyhedrin promotor RBcDNA fusion, pAcYM1/RB2.8 [i.e.,], in the bottom of direct control of a polyhedrin promotor. The array of a fusion joint is shown in the direction under drawing 1 . Moreover, the case notation under drawing 1 shows a polyhedrin promotor, and the upper case notation shows a RBcDNA array. And BamH1 linker is underlined. An arrow head shows the translation of the fusion gene using ATG (nucleotide 139) of RB. a* (+1) of drawing 1 shows the 1st A of the translation initiation codon ATG of a PORIHE drine compounds gene.
[0054] The imprint to a viral genome from the recombination plasmid of RBcDNA is a wild type Autographa by the RIPOFE cushion (lipofection). It carried out by carrying out the conte run SUFE cushion (contransfection) of the PaCym1/RB2.8DNA using a californica nuclear polyhedrosis virus DNA (BRL). Since a PORIHE drine compounds closing object was not accepted in an infected cell, the recombination virus which inactivated the PORIHE drine compounds gene by the ARERIKKU permutation with RB gene by family recombination was identified according to the plaque gestalt. Plaque purification of the virus was carried out 3 times, and the pure Baculoviridae stock was obtained. This was set to AcNPV-Y4RB.
[0055] Before the manifestation AcNPV polyhedrin promotor of the exogenous RB protein in B-2. infection insect cell determines whether Homo sapiens RB gene expression is brought about as a different-species invertebrate cell Sf9 cell is prepared. The yeast rate of 3.33 gm/l (yeastolate), insect culture-medium (Grace's insect medium)-Bull.1555 (a Texas (1987) agricultural experiment place --) of the grace which added a lactalbumin hydrolysis production object (GIBCO) and 10% of heating inactivation fetal calf serum (GIMINI), A college experiment place and TX are used and it is Sf9, i.e., Spodoptera, by the monolayer or suspension culture at 27 degrees C. frugiperda

IPLB-Sf21-AE [In vitro, 13:213-217 (1977)] was cultivated. When carrying out large-scale production of the cell lysate, the spinner culture of Sf9 cell was cultivated by the EX-CELL400 convention culture medium (J. R.Scientific) which does not contain a blood serum. Suspension culture of the malt (Molt) -4 cell of Homo sapiens T leukemia cell lineage was carried out by RPMI1640 culture medium which added the calf serum 20%. Saos-2 cell of osteosarcoma cell lineage was cultivated by the Dulbecco (Dulbecco) denaturation Eagle's medium which added fetal calf serum 7.5%.

[0056] When an AcNPV PORIHE drine compounds prow motor determined whether Homo sapiens RB gene expression is brought about as a different-species invertebrate cell, infection processing of the Sf9 cell was carried out by plaque purification AcNPV-Y4RB. 40 hours after infection, the lysate of an infected cell was collected and immunoprecipitation processing was carried out with anti--RBO-47 antigen. Next, after carrying out SDS-PAGE processing of the sample, western blotting analysis was carried out.

[0057] The pp110RB identification result in the AcNPV-Y4RB infection insect cell performed to drawing 2 and drawing 3 by western blotting analysis is shown. In the case of drawing 2 , the cell extract was prepared 40 hours after infection from the simulation infected cell (path 2), the AcNPV-Y4RB infected cell (path 3), or wild type AcNPV infection Sf9 cell (path 4). The malt -4 of Homo sapiens leukemia cell lineage was used as contrast (path 1).

[0058] Although the manifestation was accepted in the extract (path 3) of an AcNPV-Y4RB infected cell in RB protein of the same full length as a mammalian cell (path 1) when immunity blot processing was carried out by the monoclone system antibody as shown in drawing 3 , in the case of the simulation infected cell or the wild type AcNPV infected cell (paths 2 and 4), it did not accept. In order to ask for the optimal stage of RB protein production, in the case of drawing 3 , the time amount after infection was changed, and the cell extract was obtained from AcNPV-Y4 infected cell in it. Immunoprecipitation processing of the lysate was carried out with anti--RBO.47 antigen, and immunity blot processing was carried out by the pMG3-245 monoclone system antibody. p110RB of drawing 3 and pp110RB express non-phosphoryl-ized protein and phosphoryl-ized protein, respectively. After infection acted as the monitor of the production of RB protein, and asked for the optimal stage to collect cells. As shown in drawing 3 , RB protein production could be detected 24 hours after infection, and the 12 next hours increased the amount of production considerably. Protein production level was maintained all the time over about 72 hours after infection, and the bacteriolysis of a cell started it substantially 72 hours after. In order to suppress protein degradation accompanying lysis to the minimum, infected cells were usually collected about 40 hours after infection.

[0059] When detecting the manifestation of RB protein, AcNPV-Y4RB was used and infection processing of the Sf9 cell was carried out by MOI of 0.5. The cell of 5x104 was dissolved 24, 36, 48, 60, and 72 hours after from infection in the 1ml dissolution buffer solution (Naf [of Nonidet (Nonidet) P-40;1mM / of EDTA;100mM] of NaCl;50mM of NaCl of 50mM, and 7.4; 0.2% of pH, and PMSF of 1mM), respectively, at-long-intervals alignment processing was carried out for 5 minutes, and the lysate was defecated. Next, the incubation of the lysate was carried out with anti--RBO.47 antigen, and the immune precipitate was separated by SDS-PAGE 7.5%. Then, protein was moved to nitrocellulose paper and it processed with the conventional method. After leaving it in an overnight cut off state and carrying out incubation processing of the nitrocellulose paper with pMG3-245 anti--fRB monoclone system antigen for 3 hours, according to Cell and the publication of 54:275-283 (1988), it processed by the alkaline phosphatase junction goat **-mouse IgG and the chromophoric substrate.

[0060] As for the phosphoryl-ized RB gene after the nuclear normal position of B3. exogenous RB protein, and a translation, molecular weight (Mr) codes the nucleus phosphoprotein of 110,000. In order to investigate whether RB protein produced into the insect cell by Baculoviridae reached the nucleus correctly, 40 hours after infection, anti--RB0.47 antigen was used and immunity dyeing of the AcNPV-Y4RB-infection Sf9 cell was carried out. The normal position between cells of RB protein discovered into the insect cell by immunity dyeing is shown in drawing 4 and drawing 5 . The case of AcNPV-Y4RB-infection Sf9 cell is shown in the case of mock infection Sf9 cell, and drawing 5 at drawing 4 . As shown in drawing 4 and drawing 5 , the infected cell contained the unusually large nucleus. Such a condition is peculiar to the cytopathogenic effect of the Baculoviridae infection. Dyeing was not accepted when the incubation of mock infection or the wild type AcNPV infection Sf9 cell was carried out with anti--RB0.47 antigen (drawing 4). However, dyeing strong only against the nucleus of an AcNPV-Y4RB infected cell was accepted (drawing 5). When SDS-PAGE and Western blotting analyzed the nucleus and cytoplasm extract from AcNPV-Y4RB infection Sf9 cell, it has checked that exogenous RB protein mainly existed in a nucleus part.

[0061] Immunity dyeing was performed at the following process. Also in both mock infection wild type AcNPV infection and AcNPV-Y4 infection the overnight incubation of the Sf9 cell was inoculated and carried out to the chamber slide (Miles Scientific) which carried out coating of the Polly L-lysine (Sigma) 40 hours after infection. Before moving from all to the following process, the phosphoric-acid buffer sodium chloride water solution washed the slide. That is, the interstitial cell was fixed for 20 minutes for 10 minutes using the acetone (-20 degrees C) using the 0.04M phosphate buffer solution of 4% formaldehyde of ****. After carrying out the pre-incubation of the fixed cell for 10 minutes with the PBS solution of 2% of normal goat blood serum, the overnight incubation was carried out with anti--RB0.47 antigen diluted to 0.02% of triton X-100. The BIOCHINIRU-ized goat **-rabbit IgG (Canada and TAGO of Burlingame) was added after washing. 1 hour after, after carrying out the incubation of the interstitial cell for 45 minutes using AB complex joined by the horseradish peroxidase (Canada and Vector Labs of Burlingame), the incubation was carried out using the following substrate. This substrate consisted of 0.05% of 3 and 3'-diaminobenzidine tetrahydro chloride, and 0.01% of a 0.05M tris-HCl solution of H2O2. pH7.6 (Sigma). The reaction was suspended by carrying out PBS washing of the cell after 3 - 5 minutes. Next, a photograph of a cell was taken with the NIKON diamond photograph microscope (diaphotomicroscope).

[0062] The result of phosphoryl-izing of RB protein produced into the insect cell at drawing 6 and dephosphorylation is shown. 40 hours after AcNPV-Y4RB infection, a 35S-methionine or 32P-orthophosphoric acid was used, and metabolic turnover labeling of the Sf9 cell was carried out in 3 hours. Malt -4 was used as contrast and immunoprecipitation of the cell lysate was carried out using anti--RB0.47 antigen. before carrying out potato acid phosphatase (PAP) processing, (paths 1, 2, 3, and 4) were separated, SDS-PAGE (path 1', 2', 3', and 4 -- ') separated 35S- and a 32P-labeling RB protein immune complex after processing, and radioautography analyzed. Similarly, the dephosphorylation experiment was conducted using the lysate from a non-labeled cell, and western blotting analysis was performed before and after potato acid phosphatase processing (paths 5 and 6 and 5', 6').

[0063] Furthermore, the case of drawing 4 is explained continuously. Phosphoryl-ization of RB protein is produced in a multiplex serine and threonine residue, and the molecular weight heterogeneity of RB protein in SDS-PAGE is explained. In order to investigate whether phosphoryl-ization is received after RB protein produced into [Oncogene Res., 1:205-214(1989);Cell, 56:57-65 (1989)] insect cell translating, a 35S-methionine or 32P-orthophosphoric acid was used from 3 hours after infection in 40 hours, and metabolic turnover labeling of the AcNPV-Y4RB-infection Sf9 cell was carried out. Immunoprecipitation processing of the cell extract was carried out, and radioautography processing was carried out after carrying out SDS-PAGE analysis. What is necessary is just to refer to the paths 2 and 4 of drawing 6 about this point, respectively. It was parallel to this, the immunoprecipitation nature RB protein from the same extract was processed by potato acid phosphatase (PAP), and the dephosphorylation effectiveness RB protein migratory [in SDS-PAGE] was tested. The molecular weight 110,000 of 35S-labeling RB protein fell from the double band to 1-fold band after dephosphorylation (path 2' of drawing 6). And activity emitted almost completely from 32P-labeling RB protein (path 4' of drawing 6). The band change pattern as PAP processing also with the same dephosphorylation analysis by Western blotting about the lysate from a non-labeled cell which carried out infection processing by AcNPV-Y4RB was shown (the path 6 of drawing 6 , 6'). These observation result showed having phosphoryl-ized RB protein produced into the insect cell. Moreover, the molecular weight heterogeneity of RB protein accepted by SDS-PAGE from this change has been explained.

[0064] Radiation labeling and dephosphorylation analysis of Sf9 insect cell were carried out at the process shown below. 40 hours after infection, the incubation of the Sf9 during 30 minutes cell (3x106) was carried out using the 60mm pan by the methionine and the DME culture medium containing neither of a phosphoric acid which added 10% of fetal calf serum. Then, metabolic turnover labeling of the cell was carried out by adding a 0.25 mCi(s)/a ml 35S-methionine (1134 Ci/mmole, NEN) or 0.25 mCi(s)/ml 32P-orthophosphoric acid (support nothing, ICN) in 3 hours. The cell was extracted using the dissolution buffer solution (tris of 50mM - NaF [of Nonidet P-40;1mM / of EDTA;100mM] of NaCl;50mM of HCl and 7.4; 0.2% of pH, and PMSF of 1mM), and immunoprecipitation processing of the extract was carried out with anti--RB0.47 antigen.

[0065] Potato acid phosphatase (PAP and Boehringer) dephosphorylation analysis of two thirds of the immunoprecipitation RB protein from 35S- or the 32P-labeling cell lysate, and the non-labeled cell lysate was carried out. [Oncogene Res., 1:205-214 (1989)]. The incubation of the RB protein content immune complex was carried out using PAP of 1.5 units in 37 degrees C (leupeptin of MES of 20mM, and 2; 50micro [of NaCl;1MgCl(s) of mM of pH5.5;100mM] M) of reaction buffer solutions, and 60 minutes, and after the reaction, after carrying out SDS-PAGE analysis 7.5%, radioautography or Western blotting analyzed RB protein.

[0066] Infection processing of the Sf9 cell was carried out by AcNPV-Y4RB by the multiplicity of infection (MOI) of purification 1.0 of RB protein from B4. infection insect cell, and the cell lysate was obtained 40 hours after infection. The total level of RB protein discovered in the Baculoviridae system on this condition was about 17-18mg per 1l. of infection insect cell culture objects ("109 cell). About this point, **** which illuminates Table 1 3 is good.
[0067]
[Table 1]
Table [ ] 1 purification process of the recombination RB protein from the Baculoviridae infection insect cell Sum total tongue RB tongue Yield Whenever [purification] Purity Park mass Park mass (%) (%)
(mg) (mg) A cell extract 670a 16c 90c 1.0 times 2.3pMG3-245 immunity affinity column 13.5b 12.9d 72 cds 41.3 times 95 Protein quantum c. western blotting by the protein quantum b. micro BCA (PIERCE) by the approach and spectrophotometry of a. Bradford (Bradford) (Bio-RAD) And the protein quantum by the protein quantum d. Coomassie-brilliant-blue-staining method by the density measurement method, and the density measurement method [0068] As shown in Table 1, 90% of discovered RB protein (16mg) was accepted in the supernatant after cell division, and 10% had stopped at the insoluble fraction. Since 2.3% of all cell protein was formed, RB protein was easily detectable from the cell lysate. About 135mg protein was recoverable from the alkaline eluate of a column after one-step immunity affinity chromatography purification. In order to investigate the purity of elution RB protein, SDS-PAGE and Coomassie brilliant blue staining analyzed the eluate aliquot equivalent to 2.5x105 cell.

[0069] What is necessary is just to refer to drawing 7 which shows immunity affinity chromatography purification of pp110RB about this point. After analyzing the aliquot (equivalent to 2.5x105 infected cell) of eluate with the electrophoresis method by 10%SDS-poly AKURIRO amide gel from the rough lysate from mock infection Sf9 cell (path 1) or AcNPV-Y4RB infection Sf9 cell (path 2) of 1x105, and pMG3-245 anti--RB immunity affinity chromatography, it analyzed by the Coomassie-brilliant-blue-staining method. The arrow head has shown RB protein of anticipation molecular weight.
[0070] When judged by the density measurement method, the above-mentioned one-step purification process is effective, and RB protein was able to be obtained by 41.3 times whenever [95% / of purity / (drawing 7 , path 3), 72% / of yield /, and purification] (Table 1).
[0071] Some modification was added although the immunity affinity column was constituted according to the publication of Shneider, etc. ****, etc. [J. Biol.Chem., 257:10766-10769(1982);Virology, 144:88-100(1985)]. After filling up the Bio-Rad (Bio-Rad) column with 2ml protein G-agarose (Genex) and washing by 0.01-N HCl, the joint buffer solution (sodium acetate of 0.1M, NaCl of pH5.0;0.1M) washed. It joined together by adding the anti--fRB monoclonal antigen (pMG 3-245) of 2mg [15] to a column. Next, the boric acid buffer solution of 0.1M washed the column widely, and the bead was re-suspended in the 20ml buffer solution. Dimethyl PIMERIMI date dihydrochloride (Sigma) was added so that the last concentration might be set to 40mM(s), mixture was stirred at the room temperature for 1 hour, and crosslinking reaction was performed. after washing and a room temperature -- for 10 minutes, 20ml solution of ethanolamine-0.1M boric acid buffer solutions of HCl of 40mM, and pH8.0 -- it came out and the residual reactivity radical of a bead was intercepted. then, the glycine of 0.2M and pH2.3 -- it came out and the column was washed, and it saved in this solution until it neutralized and was needed with tris buffers (tris-HCl of 50mM, EDTA [of pH7.4;100mM] of NaCl;1mM of PMSF:1mM). When OD280 of the first monoclonal antigen sample and OD280 of the flow fraction in the following process were measured, about 10mg pMG 3-245 had combined with the protein G-agarose which is 2ml.
[0072] About RB protein, two biochemical properties are described despite specific complex formation current [with B5.DNA avidity and an SV40 T antigen]. That is, it is one of them that DNA is combinable as a peculiar property, and it is one more [Nature, 329:642-645 (1987)] and that how many kinds of the neoplasm protein and the specific complex of that DNA tumor virus can be formed [Cell, 54:275-283,;(1988) Science, 243:934-937 (1989);Nature (London), 334:124-129 (1988)]. About these [which are participating in proteinic biological functions] two well-known biochemical properties, RB protein refined from the Baculoviridae infection insect cell was tested.
[0073] A south Western DNA-joint measuring method is shown in drawing 8 and drawing 9 . The purification trpE-RB fusion protein of 6microg and purification Baculoviridae manifestation pp110RB were applied to SDS-PAGE 10%. In the case of the joint measuring method shown in drawing 8 , the Coomassie-brilliant-blue-staining method was used, and, in the case of the joint measuring method shown in drawing 9 , the electric imprint of the parallel

gel was carried out at nitrocellulose paper. Next, the incubation of the blot was carried out by the 32P-labeling DNA fraction, and radioautography analysis was carried out. In the case of drawing 8 and drawing 9 , it is as follows. Path 1:RB19-22; path: -- RB23-27; -- path 3:RB19 -- the purification RB protein from a -27; path 4:AcNPV-Y4RB infection insect cell.

[0074] In the case of drawing 8 and drawing 9 , DNA binding was measured by south Western analysis. In this case, SDS-PAGE separated the trpE-RB fusion protein of tales doses, and the purification RB protein from an insect cell 10%. The amount of the protein by which loading was carried out was calculated by the Coomassie-brilliant-blue-staining method (drawing 8). In the experiment which used another parallel gel, after carrying out the electric imprint, the incubation was carried out to the nitrocellulose membrane by the 32P-labeling DNA. Radioautography analyzed next DNA combined with protein (drawing 9). The affinity to DNA of the fusion protein RB 19-27 with which the main domain interacts with DNA is 20 times as high as two fields RB 19-22 and RB 23-27. About this point, the path 3 of drawing 9 could be compared with paths 1 and 2, and purification full length RB protein showed strong DNA avidity like RB 19-27 (drawing 9 , path 4). The DNA avidity of the purification RB protein from an insect cell is elution which continues after that from a column even if based on maintenance of the protein by the DNA cellulose. - It has proved also by NaCl- of about 400 mM(s).

[0075] Purification of pp100RB from an infection insect cell was carried out as follows. By MOI of 1.0, using AcNPV-Y4RB, infection processing was carried out and suspension culture of the Sf9 cell was carried out (1X166 cell / ml, 1000ml). 40 hours after infection, low-speed centrifugal separation pelletizes and washes a cell, and it is tris of 50mM(s). - It re-suspended in the extract buffer solution containing NaCl;10% the leupeptin of PMSF;25microg of DTT;1mM and 50 units of NP-40;1mM of glycerol (v/v);1mM of HCl and 7.4; 0.2% of pH / aprotinin of ml. [of EDTA;100mM] After carrying out an incubation for 15 minutes in Hikami, centrifugal (for 10,000xg, 4-degree-C, and 10 minutes) clarification processing of the sample was carried out, and RB content supernatants were collected. As mentioned above, immunity affinity chromatography of pp110RB was carried out in the column of 2ml capacity containing the anti--fRB monoclonal antigen (pMG 3-245) combined with protein G-agarose. After letting a supernatant pass 4 times in a column, the following solution was used in the amount of 200 bed capacity, respectively, and the column was washed continuously. The dissolution buffer solution, the dissolution buffer solution containing NaCl of 500mM, and a penetrant remover (PMSF;10% glycerol [of 200mM / of NaCl;1mM] of EDTA;1mM of DTT;1mM). Next, binding protein was eluted from the column with the alkaline elution buffer solution containing PMSF of EDTA;1mM and 10% of glycerol of NaCl;1mM of the triethylamine of 2mM, and pH10.8;200mM. 1ml fractions are collected and it is tris of 1M immediately. - It neutralized by 1/12 capacity of HCl (pH7.5), and saved at -70 degrees C in 10% glycerol.

[0076] When refining pp110RB from an infection insect cell, after calculating the total amount of protein, the south Western DNA binding measuring method and the SV40 T antigen joint measuring method were enforced.

[0077] The amount of all the protein of the elution fraction of an immunity affinity column was calculated with the micro-BCA measuring method (PIERCE). SDS-PAGE analyzed the elution protein sample next, and the Coomassie-brilliant-blue-staining method and the degree were asked for the amount of RB protein of eluate by the density measurement method. The total amount of protein of a cell extract was measured by the approach (Bio-Rad) of Bradford. [Anal.Biochem., 72:248-254 (1976)]. In order to carry out the quantum of the RB protein of the cell lysate, after using the purification RB protein diluted in order as a criterion and carrying out Western blotting, density measurement compared band strength. What is necessary is just to refer to Table 1 about this point.

[0078] Protein blotting was carried out with the conventional method. According to Bowen's (Bowen) etc. publication, the incubation of the blot was carried out by the radiation labeling DNA. [Nuleic Acids Res.8:1-21 (1980)]. This was performed at the room temperature. After rinsing the blot quickly, it washed 3 times by the urea of 6M, and 0.2% of NP-40 (all are 20 minutes), and washing (all are 30 minutes) was succeedingly carried out 4 times with the DNA binding buffer solution (NaCl;0.2% BSA;0.2% Ficoll400 and 0.2% of polyvinyl pyrroline of EDTA;50mM of tris-HCl of 10mM, and pH7.0;1mM). Next, the incubation of the blot was carried out for 30 minutes in the DNA binding buffer solution including the 32P-labeling DNA. By EcoR1, line-ized pGEM1DNA was labeled by the alpha-32P deoxy nucleotide (Amersham, >3000 Ci/mmol) by the random priming, and it was used as a probe. Washing of the blot was carried out 3 times (all are 10 minutes) with the DNA binding buffer solution after hybridization, it was air-dry and radioautography analyzed. TrpE-RB fusion protein was included as contrast. Each trpE-RB fusion protein was named according to the exon of RB gene which protein contains. That is, RB 19-22, RB 23-27, and RB 19-27 occupy the pp110RB field of an exon 19-22 (amino acid 612-775), an exon 23-27 (amino

acid 776-928), and an exon 19-27 (amino acid 612-928), respectively.
[0079] Immunity chromatography purification of the SV40 T antigen was carried out from Ad-SVX1-infection 293 cell. [J. Virol., 53:1001-1004(1985);Cold Spring Harbor Press., Cold SpringHarbor, NY, pp.187-192(1982)]. And anti--T monoclonal PAG419 antigen was obtained from Oncogene. The well-known complex formation measuring method was enforced. However, some modification was added. That is, the Baculoviridae manifestation RB protein of 800ng was mixed with the 1ml EBD buffer solution (NaCl of tris-HCl of 50mM, and pH8.0;120mM, and 0.5% of Nonidet P-40) containing PMSF of 1mM, 25microg [/ml] leupeptin and 50 units / aprotinin of ml. The purification T of 800ng(s) was added to mixture, and the incubation was carried out in Hikami during 90 minutes. anti- -- using either of -RB0.47 or PAB419 antigens, immunoprecipitation of the aliquot of mixture was carried out and Western-blotting analysis was carried out. The blot made it react with pMG 3-245 and PAB419 in order. After carrying out an incubation with the alkaline phosphatase junction goat **-mouse IgG, coloring processing of the blot was carried out using the color-enhancing substrate.
[0080] In order to test the capacity for purification RB protein to be able to form an SV40 T antigen and specific complex, equivalent RB protein and an equivalent T antigen were mixed, and immunoprecipitation of the aliquot of mixture was carried out using anti--RB0.47 antibody or -anti-T antigen PAB419.
[0081] In relation to this point, the complex which Baculoviridae manifestation RB protein and an SV40 T antigen form in drawing 10 is shown. Purification Baculoviridae manifestation RB protein was mixed with the purification T antigen by in vitro one. next, the mixture of this aliquot -- PAB419 (path 2) -- anti- -- immunoprecipitation was carried out by either of -RB(s)0.47 (path 3), and Western blotting analyzed. Paths 1 and 4 show the purification Baculoviridae manifestation RB protein which carried out immunoprecipitation by the purification SV40 T antigen which carried out immunoprecipitation by PAB419, respectively, and anti--RB0.47 antibody.
[0082] As shown in drawing 10 , when the T antigen was mixed with RB protein by in vitro one, PAB419 not only coimmunoprecipitates with RB protein, but (the path 2), and T and anti--RB0.47 antigen coimmunoprecipitated (path 3). It is proved [data / these] that RB protein from the Baculoviridae infection insect cell can form an SV40 T antigen and specific complex.
[0083] Since it became clear that the nuclear transformation purification protein of B6. purification RB protein had the two well-known biochemical properties of RB by in vitro one, it investigated about the in vitro behavior of purification protein next. The cytoplasm of Saos-2 cell with the osteosarcoma cell lineage without an exon 21-27 which codes C terminal cutting RB protein (p95), including a deficit RB gene was injected with purification RB protein. [Proc.Natl.Acad.Sci.U.S.A., 87:6-10 (1990)]. The antibody orientated this protein to the cytoplasm in consideration of the fact of going to the C terminal of RB protein, in the small quantity of extent which is not accepted by anti--RB0.47 antibody to be used. Immediately after injection, the cell was fixed and it analyzed by the immunity staining technique. The nuclear normal position of generation RB protein after carrying out a microinjection to drawing 11 to the cytoplasm of Saos-2 cell is shown. The cell was injected with generation RB protein and it analyzed by the immunity staining technique. The arrow head shows among drawing that dyeing of the nucleus after a microinjection is strong compared with the non-injecting cell.
[0084] As shown in drawing 11 , compared with a non-injecting cell, dyeing of the nucleus after a microinjection is strong (arrow head), and it is shown that injection protein was immediately conveyed to the nucleus. since RB protein is known as a nucleoprotein -- the nuclear normal position of after a microinjection and purification protein -- immediately -- and although cut correctly, this has suggested that this protein is activity in in vivo one.
[0085] In the case of the microinjection, purification RB protein was dialyzed so that the last concentration might become [ml] the injection buffer solution containing DTT of EDTA;0.1mM and 2% of glycerol of KCl;0.1mM of tris-HCl of 20mM, and pH7.4;10mM in 0.5mg /. The capillary glass tube needle (Eppendorf) was used and the microinjection of the Saos-2 cell which grew on the glass CHANN bar slide was carried out by the usual approach. The micro manipulator and opposition contrast microscope (NIKON make) of Eppendorf which attached the vacuum pressurizer were used, and micromanipulation of the capillary tube was carried out, respectively, and the microinjection process was visualized. After the microinjection, the cell was immediately fixed with the 0.04M phosphate buffer solution solution (pH7.4) of formaldehyde 4%, and it analyzed by the immunity staining technique.
[0086] As explained more than B6. epitome, it has proved that the bottom Homo sapiens retinoblastoma gene production object of the transcriptional control of the Baculoviridae polyhedrin promotor could be efficiently discovered. Since RB protein had the misgiving which it not only blocks growth of a cell, but shows "toxicity" to it depending on the case, it was thought that the attempt which has discovered RB protein on high level for a long time was difficult. The imprint of the foreign gene from a polyhedrin promotor is produced in the second half of

infection, and cutoff of production of an extracellular virus particle, a cell, and a great portion of virogene continues next. Therefore, when superfluous production is carried out, the Baculoviridae insect cell lineage is advantageous to composition of protein, such as RB protein which has that it is harmful to cell growth. Another advantage of this system is in the similarity of an insect and the protein synthesis path of a mammalian cell.
[0087] Moreover, it also becomes clear that RB protein reaches the nucleus of an insect cell correctly, and this connotes that the nuclear normal position signal of mammalian is recognized by the insect cell. Although glycosylation of the recombination protein in the Baculoviridae manifestation system is restricted to 0-association of a high mannose mold, and an N-joint oligosaccharide, suitable phosphoryl-ization of outpatient department protein is reported about c-myc and the manifestation of HTLV-Ip40x (J. Virol.). Although RB protein was phosphoryl-ized from before, not carrying out glycosylation was known. This is a reason for production of functional RB protein with the suitable Baculoviridae manifestation system.
[0088] As indicated on these specifications, RB protein produced into the infection insect cell is phosphoryl-ized after a translation, therefore can specialize a multiplex band by Western-blotting analysis completely like the case of RB protein of Shinsei mammalian. However, when seeing from band strength and it compares with hyper-phosphoryl-ized RB protein, phosphoryl[non-]-izing and a phosphoryl-ized gestalt are superior. Since this phenomenon's being reflection of the cell cycle condition of the population at the time of viral lytic infection or a lot of exogenouses RB exist in a cell the place to current, it is not known whether it is because phosphoryl-izing of the protein by the insect kinase is only inadequate. In order to determine the same thing **** as the case of phosphoryl-ized PATANGA mammalian protein, and accuracy, it is necessary to map the phosphoryl-ized location of RB protein in a precision.
[0089] The total level of the recombination RB protein discovered in the Baculoviridae system is about 17 to 18 mg per 1l. (109 cells) of infection insect cell culture objects. This manifestation level is equivalent to other mammalian protein which this system generates. For example, in the case of interleukin 2, it is 10-20 mg/l [The Banbury Report.Fields, B., Martin, M.A.& Kamley, D. (ed.), 22:319-328 (1985), Cold SpringHarbor Laboratory Press, Cold SpringHarbor]. moreover, [Oncogene, 4:759-766(1989)]. which is 4-5 mg/l in P210 BCR-ABL -- if a recombination transition vector including the 5 'PORIHE drine compounds 5 without deficit united with RBcDNA which removed most non-coding fields' non-translated field is used, high RB protein manifestation level can be made still higher. This RBmRNA array is G+C Rich highly, and is a factor advantageous to forming stable secondary structure. These structures are considered to make translation effectiveness of Correspondence mRNA low when it is before an initiation codon. about the in vitro translation of RBmRNA, it proves [become / 10 times / high] from 5 times by permuting RB5' a non-translated array by it of an alfalfa mosaic virus (AMV) RNA 4 or beta globulin mRNA -- having -- this -- RB5' -- [EMBO J. (1990)] which is what suggests having a bad influence on the translation of a non-coding array potentially. Moreover, if 5' non-translated array with a long foreign gene exists, it will also have become clear that the recombination PORIHE drine compounds manifestation in the Baculoviridae system is influenced. since A+T rich nature of a polyhedrin promotor is very strong, before inserting in a transition vector as a conclusion -- long -- and G+C -- rich 5' non-coding array should be trimmed from RBcDNA, and the pp110RB manifestation should be optimized.
[0090] In order to suppress the denaturation of the protein under purification to the minimum, several kinds of different protocols were tested about elution of RB protein from an affinity column. Since many are not known so much about the biological functions and the biochemical property of RB protein, it is only two parameters that it can be used as a scale of the integrity of purification protein. That is, it is complex formation with DNA avidity and an SV40 T antigen. In addition, the conditions which maintain a proteinic biochemical property were elution conditions which use the triethylamine of 20mM(s) by pH10.8 in this invention. Although purification protein carried out the nuclear normal position immediately from the cytoplasm after the microinjection, it is proved [this / protein / this] that activity is shown under these elution conditions. When protein was eluted by extreme pH (triethylamine of the glycine of 200mM, pH 2 and 3, or 100mM(s), pH11.5), the inclination for protein to denaturalize was accepted. That is, the two above-mentioned activity fell. This is clear also from insoluble floc having formed after prolonged preservation.
[0091] According to the former report, although [non-phospho RURIRU-ized RB protein] the SV40 T antigen of D2C2 cell which is the stable transformant by the SV40 T antigen of nephrocyte network valve flow coefficient1-P of an ape is combinable, it was found out that the HIPOHOSUHORIRU-ized gestalt of [Cell, 56:57-65 (1989)] and RB protein of a certain kind can also form an SV40 T antigen and complex. This has been checked with sufficient repeatability, when in vitro mixing of the purification RB protein [from a T antigen and an AcNPV-Y4RB infection

insect cell] or malt -4 lysate was carried out. The same phenomenon was accepted also when complex was formed by in vivo one using a Cos cell (drawing 10). Since phosphoryl-ization of RB protein is changed in a cell cycle in the case of phase singularity and the complex formation between RB and viral neoplasm protein is participating in the transformation activity of these DNA tumor virus, the relevance of HIPOHOSUHORIRU-ized RB protein and an SV40 T antigen should be solved someday.

[0092] RB protein which does not have a meltable deficit when the Baculoviridae insect cell lineage is used, as explained above and which can also predict activity suddenly -- an owner -- it is a big advance for future biochemical and research of a biophysical property of RB gene production object that it can be used in a meaningless amount. They are analysis of the cell protein in relation to the case considered, three-dimension-structure research of RB protein using separation and X-ray crystallography of the specific DNA array with which these interact, etc. Moreover, the elucidation of the biological functions of the retinoblastoma gene in cancer control should also progress. About the intervention to the cell growth of RB and differentiation which were directly tested by the microinjection considered, it is inquiring on the current energy target.

[0093] The notation used on these specifications is explained below. cDNA -- complementary DNA and kd -- KIRODARUTON and kb -- a kilobase and SDS -- for Nonidet P-40 and MES, sodium salt (2-[N-morpholino] ethane sulfonic acid) and MOI are [a sodium dodecyl sulfate and PAGE / polyacrylamide gel electrophoresis and NP-40 / relative molecular weight and PAP of the multiplicity of infection and Mr] potato acid phosphatase. The protein production object shown by "pp110RB" is the same protein production object as "ppRB110."

[0094] It is included by the pneuma and the range which various kinds of modification is possible and all indicated to the claim although this invention has been explained about the specific example. That is, this indication does not mean a limit.

[Translation done.]

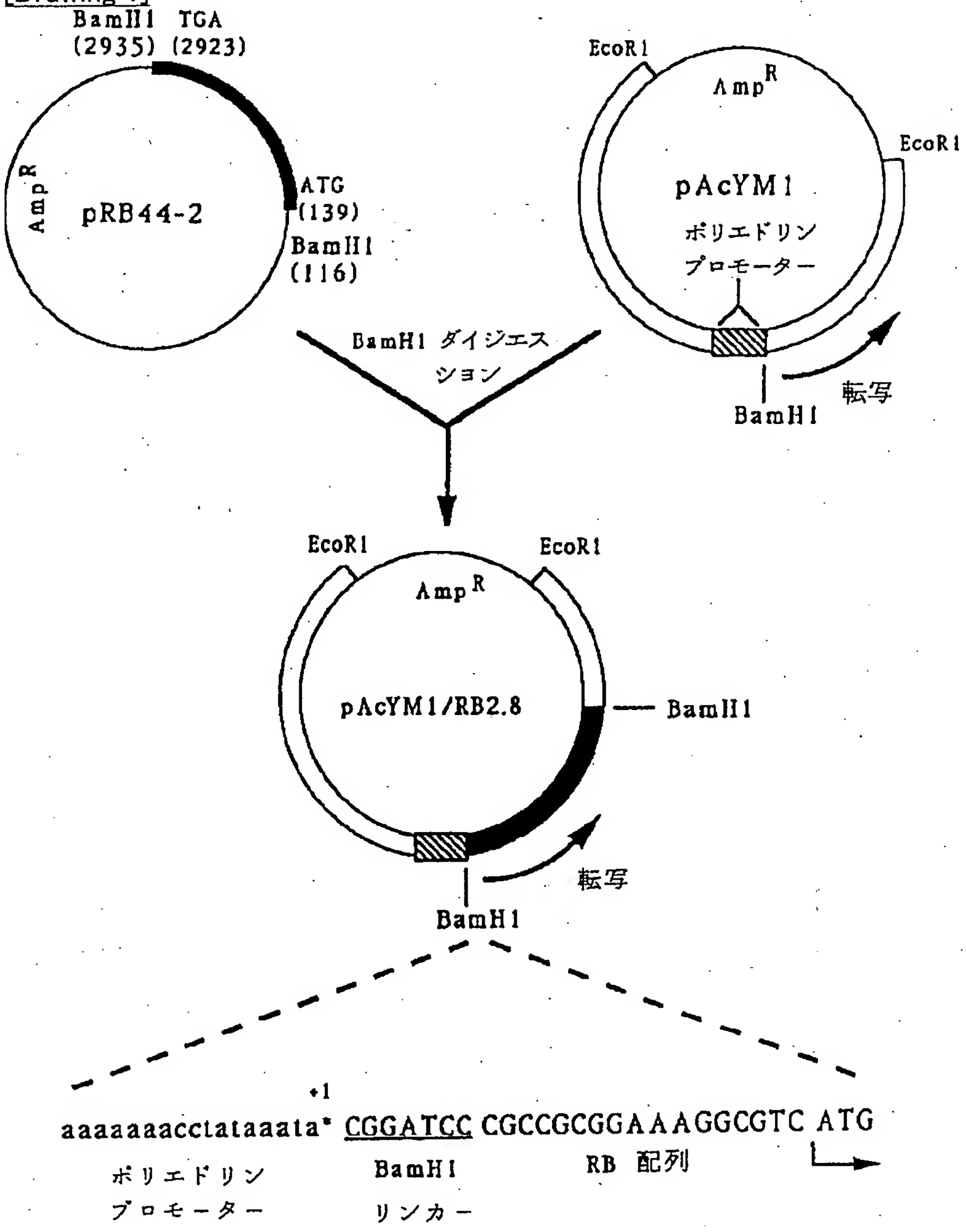
DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] It is the schematic diagram showing the configuration of a Baculoviridae manifestation vector which carries out pp110RB composition.
[Drawing 2] It is the western blotting of a ppRB infection insect cell.
[Drawing 3] It is the western blotting which identifies the cell extract extracted from the infected cell even 72 hours after infection.
[Drawing 4] It is the microphotography in which the normal position between cells of RB protein is shown.
[Drawing 5] It is the microphotography in which infection Sf9 cell is shown.
[Drawing 6] It is the radioautograph which shows phosphoryl-izing of RB protein and the dephosphorylation analysis result in an insect cell.
[Drawing 7] pMG 3-245 -- anti- -- the electrophoresis analysis of the rough lysate from -RB, infection Sf9 cell, and eluate is shown.
[Drawing 8] The fusion protein and the south Western DNA binding measuring method of Baculoviridae manifestation pp110RB which were applied to SDS-PAGE and a Coomassie-brilliant-blue-staining method 10% are shown.
[Drawing 9] It is the radioautograph of the blot from parallel gel to the gel used in order to create drawing 8 , and the incubation of this blot is carried out by the 32P-labeling DNA fragment.
[Drawing 10] It is the chromatogram which shows complex formation with Baculoviridae manifestation RB protein and an SV40 T antigen.
[Drawing 11] It is the photograph in which the purification RB protein nucleus normal position after the microinjection of cell ****** of Saos-2 cell is shown.

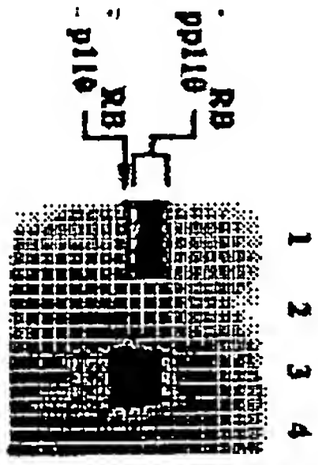
[Translation done.]

DRAWINGS

[Drawing 1]



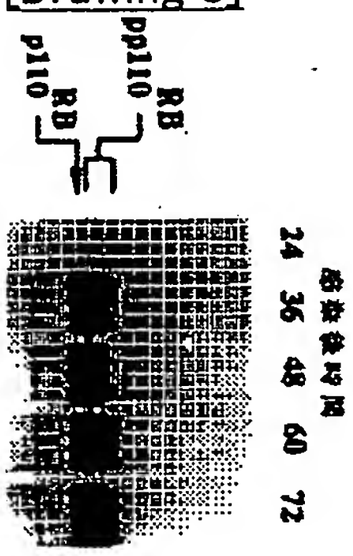
[Drawing 2]



図面代用写真

写　真

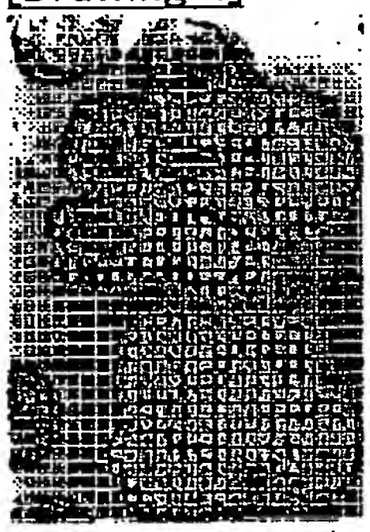
[Drawing 3]



図面代用写真

写　真

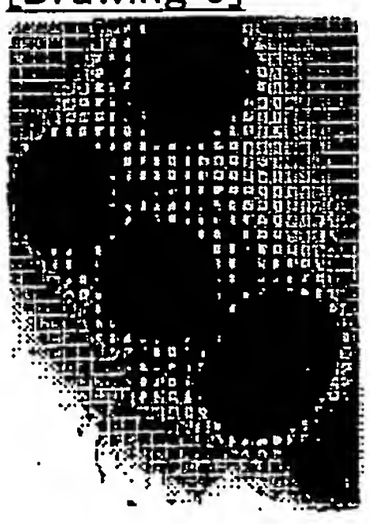
[Drawing 4]



図面代用写真

写　真

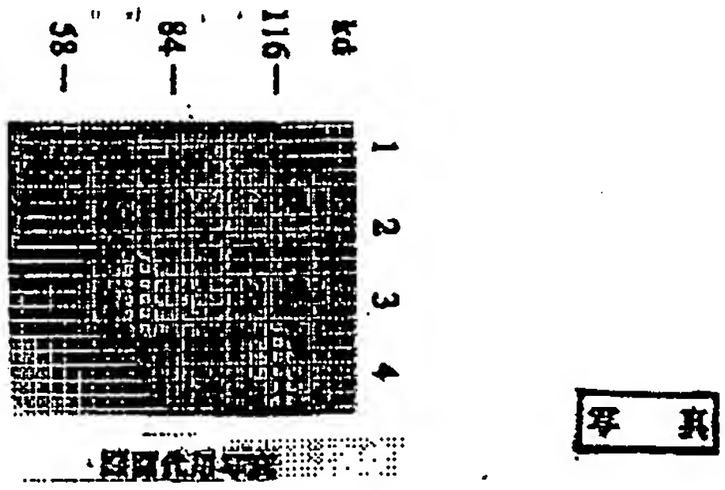
[Drawing 5]



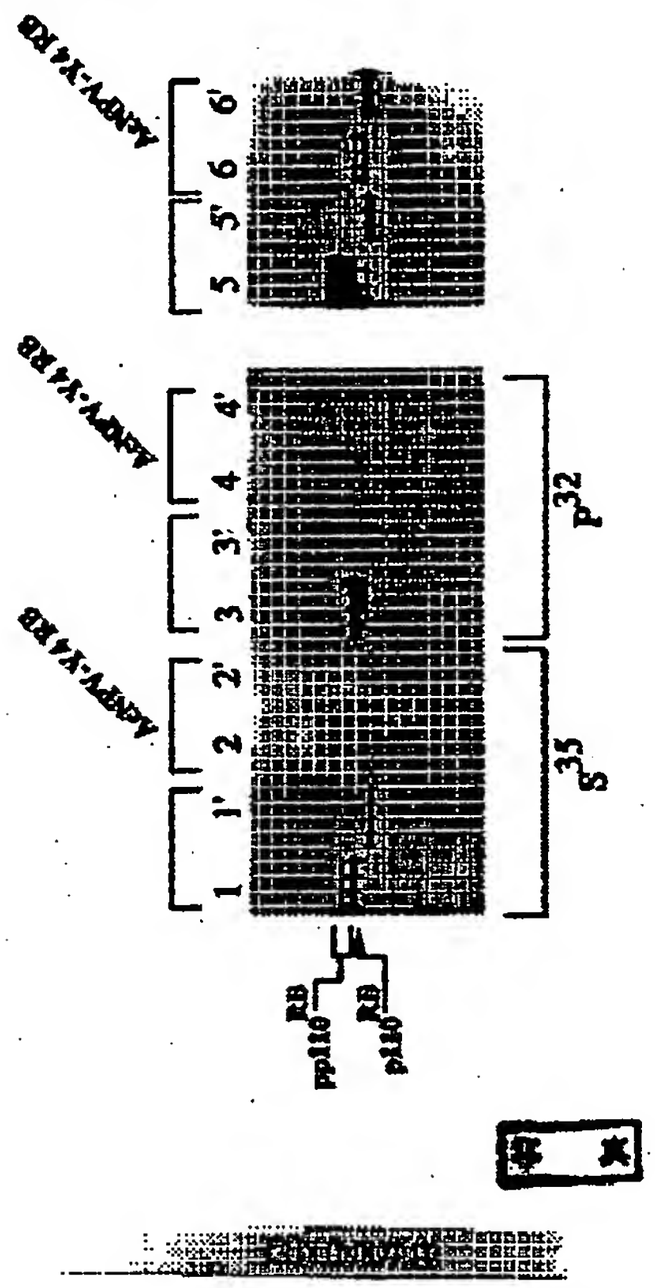
図面代用写真

写　真

[Drawing 8]

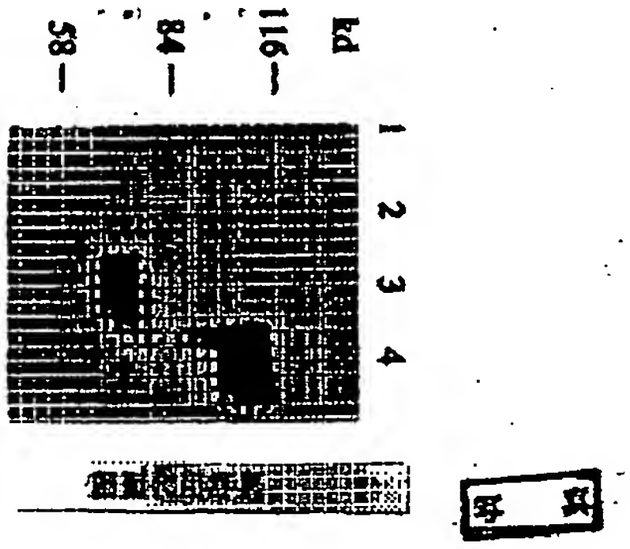


[Drawing 6]

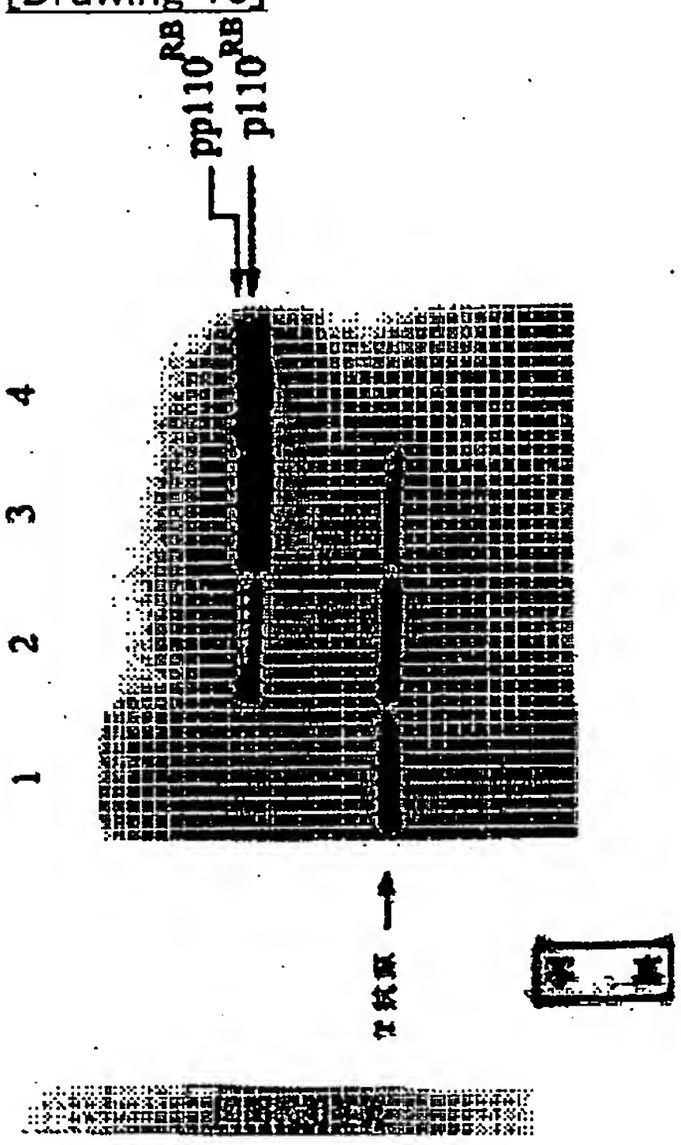


[Drawing 7]

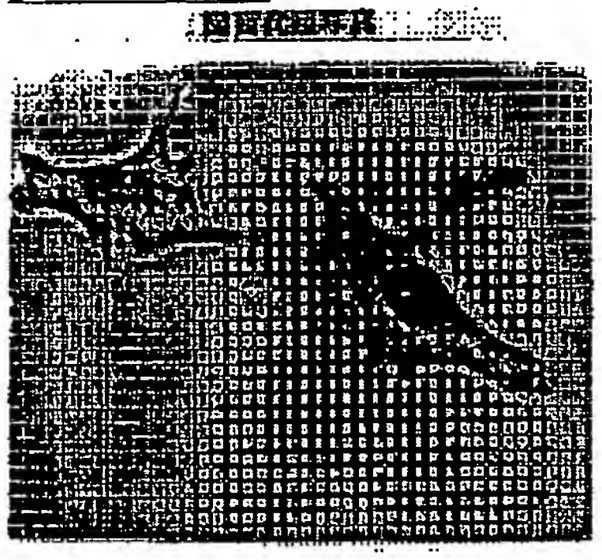
[Drawing 9]



[Drawing 10]



[Drawing 11]



[Translation done.]

* NOTICES *

WRITTEN AMENDMENT

[Procedure revision]
[Filing Date] August 18, Heisei 5
[Procedure amendment 1]
[Document to be Amended] Specification
[Item(s) to be Amended] Easy explanation of a drawing
[Method of Amendment] Modification
[Proposed Amendment]
[Brief Description of the Drawings]
[Drawing 1] It is the schematic diagram showing the configuration of a Baculoviridae manifestation vector which carries out pp110RB composition.
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[Drawing 11] It is the photograph in which the purification RB protein nucleus normal position after the microinjection of cell ****** of Saos-2 cell is shown.

[Translation done.]

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Ａ６１Ｋ　37/02	ＡＤＵ	8314－4C			
Ｃ０７Ｋ　13/00		8517－4H			
		8931－4B	Ｃ１２Ｎ　15/ 00	Ａ	
		9281－4B	5/ 00	Ｂ	

審査請求　有　請求項の数32(全 17 頁)　最終頁に続く

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(22)出願日　平成３年(1991)７月15日

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(32)優先日　1990年７月16日
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最終頁に続く

(54)【発明の名称】　遺伝子タンパク質産生物及び細胞治療法

(57)【目的】　遺伝子タンパク質産生物の産生方法の提供。
【構成】　遺伝物質の昆虫細胞への導入によって所望ポリペプチドを実質的な量で産生する方法。適当なベクターを使用して、例えば、クローン化遺伝子、遺伝子フラグメント、あるいはその誘導体をホスト細胞に導入することによって高純度のタンパク質を実質的な量で、高レベルで産生できる。

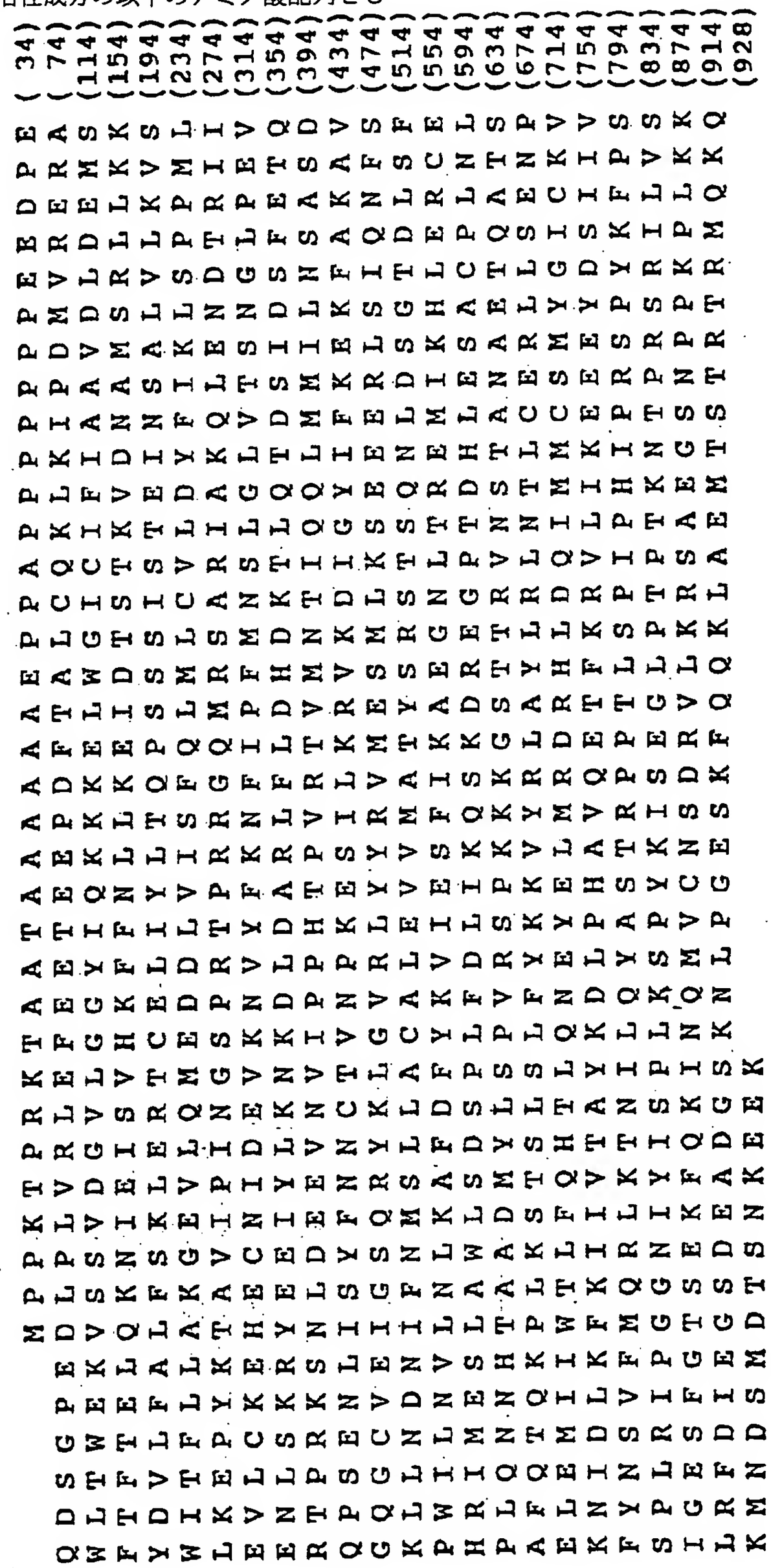
【特許請求の範囲】
【請求項１】　所定の遺伝子に関係する実質的に精製された遺伝子産生ポリペプチドを産生する方法において、該遺伝子にヌクレオチド配列に実質的に対応するヌクレオチド配列をもつｃＤＮＡを搬送するベクターを選択し、
該ＤＮＡを該ベクターに挿入し、
該ベクターを細胞培養物に導入して、該ポリペプチドを伝搬する発現系を形成し、
該細胞を成長させ、
該細胞を分裂させ、
該細胞から該ポリペプチドを抽出し、そして該ポリペプチドを精製することからなるポリペプチド産生方法。
【請求項２】　該ベクターがウィルスである請求項１に記載の方法。
【請求項３】　該ウィルスがバキュロウイルスである請求項１に記載の方法。
【請求項４】　該ウィルスがＡｕｔｏｇｒａｐｈａ　ｃａｌｉｆｏｒｎｉｃａ核多角体病ウィルスである請求項１に記載の方法。
【請求項５】　該ｃＤＮＡがポリヘドリン遺伝子プロモーター特性をもつ請求項１に記載の方法。
【請求項６】　該ｃＤＮＡがポリヘドリン遺伝子のプロモーターをもち、そして該クローン化ｃＤＮＡを該プロモーターの下流に挿入する請求項１に記載の方法。
【請求項７】　該細胞が真核生物細胞である請求項１に記載の方法。
【請求項８】　該細胞がＳｐｏｄｏｐｔｅｒａ　ｆｒｕｇｉｐｅｒｄａ細胞である請求項１に記載の方法。
【請求項９】　該精製を免疫親和クロマトグラフィー法によって実施する請求項１に記載の方法。
【請求項１０】　該ｃＤＮＡを真核生物遺伝子から誘導する請求項１に記載の方法。
【請求項１１】　該ｃＤＮＡを真核生物癌抑制遺伝子から誘導する請求項１に記載の方法。
【請求項１２】　該ｃＤＮＡを網膜芽腫遺伝子から誘導する請求項１に記載の方法。
【請求項１３】　所定の遺伝子に関係する遺伝子産生ポリペプチドを発現するポリペプチド発現系において、搬送用ベクターと細胞培養物からなるポリペプチド発現系。
【請求項１４】　該ベクターがウィルスである請求項１３に記載の発現系。
【請求項１５】　該ベクターがバキュロウイルスである請求項１３に記載の発現系。
【請求項１６】　該ベクターがＡｕｔｏｇｒａｐｈａ　ｃａｌｉｆｏｒｎｉｃａ核多角体病ウィルスである請求項１に記載の系。
【請求項１７】　該細胞培養物の細胞が昆虫細胞である請求項１３に記載の系。

【請求項１８】　該細胞がＳｐｏｄｏｐｔｅｒａ　ｆｒｕｇｉｐｅｒｄａ細胞である請求項１３に記載の系。
【請求項１９】　所定の遺伝子に関係し、かつ該遺伝子にヌクレオチド配列に実質的に対応するヌクレオチド配列をもつｃＤＮＡを搬送するベクターを選択し、
該ＤＮＡを該ベクターに挿入し、
該ベクターを細胞培養物に導入して、該ポリペプチドを伝搬する発現系を形成し、
該細胞を成長させ、
該細胞を分裂させ、
該細胞から該ポリペプチドを抽出し、そして該ポリペプチドを精製することからなる方法によって得た実質的に精製されたポリペプチド。
【請求項２０】　網膜芽腫遺伝子から該ｃＤＮＡを誘導することを含む請求項１９に記載のポリペプチド。
【請求項２１】　欠損癌抑制遺伝子、突然変異性癌抑制遺伝子をもつ、あるいは遺伝子をもたない細胞を治療する方法において、
該欠損あるいは突然変異性遺伝子を、あるいは遺伝子のないことを確認し、
該遺伝子の、天然産生遺伝子又はそのクローンである欠損のない対部分を求め、そして対部分の遺伝子のタンパク質産生物を産生することからなる治療方法。
【請求項２２】　該搬送が該タンパク質産生物を該細胞に顕微注射することを含む請求項２１に記載の方法。
【請求項２３】　該精製が該タンパク質の緩衝液への透析を含む請求項２１に記載の方法。
【請求項２４】　該緩衝液がトリスーＨＣｌ、ＫＣｌ、ＥＤＴＡ、ＤＴＴ及びグリセロールを含む請求項２１に記載の方法。
【請求項２５】　該トリスーＨＣｌのｐＨが７．４である請求項２４に記載の方法。
【請求項２６】　該緩衝液が１部のＤＴＴ、１部のＥＤＴＡ、１００部のＫＣｌ及び２００部のトリスーＨＣｌを含む請求項２３に記載の方法。
【請求項２７】　該確認がタンパク質産生物の有無を調べ、かつタンパク質産生量を測定する請求項２１項に記載の方法。
【請求項２８】　該測定が該タンパク質に特異的な抗体を使用して、該抗体と該タンパク質の免疫複合体が形成するかどうかを決定することを含む請求項２１に記載の方法。
【請求項２９】　活性成分としてのポリペプチドと生理学的に適当な担体とからなる薬剤組成物。
【請求項３０】　該ポリペプチドがｐｐ１１０^{RB}である請求項２９に記載の薬剤組成物。
【請求項３１】　乳癌抑制遺伝子、ヴィルムス腫瘍抑制遺伝子、ベックウィズーヴィーデマン症候群抑制遺伝子、遷移性細胞腫瘍抑制遺伝子、神経芽腫抑制遺伝子、小細胞肺癌抑制遺伝子、腎細胞腫抑制遺伝子及び結腸直

腸癌抑制遺伝子のタンパク質産生物から該活性成分を選択する請求項２９に記載の薬剤組成物。

【請求項３２】　該活性成分の以下のアミノ酸配列をもつ請求項２９に記載の薬剤組成物。

【化１】



【発明の詳細な説明】

【０００１】

【産業上の利用分野】本発明は全体的には遺伝子タンパク質産生物の産生方法及び細胞を治療して腫瘍形成を抑制するための細胞療法に関する。本発明はまた細胞療法及び腫瘍形成を抑制する細胞治療法に関する。本発明は政府助成（認可番号ＥＹ０５７５８）発明であり、全国保険協会（ｔｈｅ　Ｎａｔｉｏｎａｌ　Ｉｎｓｔｉｔｕｔｅ　ｏｆ　Ｈｅａｌｔｈ）及びカルフォルニア大学と共同して達成した発明である。従って、本発明については政府にも一定の権利がある。

【０００２】

【従来技術及びその課題】ヒトや動物の組織についてはかなり詳しい研究があり、数多くの欠損が細胞レベルで確認されている。これら欠損の多くは遺伝的な原因をもつもので、大半が欠損遺伝子によるものである。欠損は点突然変異等を原因とすることがあり、この結果遺伝子自体のヌクレオチド配列が分断したり、異常な変化を示すことがある。機能不全遺伝子があると、最終的には遺伝子タンパク質産生物を産生できなくなるか、それ自体が欠損した遺伝子タンパク質産生物を産生することになる。

【０００３】ヒト・動物のいずれについても欠損遺伝子を同定する場合には、遺伝子療法を適用することができる。この場合には、クローン化遺伝子を治療すべき細胞の細胞核に導き、異常な遺伝物質または欠損遺伝物質を矯正すればよい。この方法に使用される物質は製造コストが高く、複雑精巧な実験装置を必要とし、また複雑精巧な遺伝子操作技術の適用を必要とする。このような技術は一般には利用できず、従って利用できるのは大部分が比較的少数の高度に精巧な分子遺伝子操作を扱う実験室に限られている。

【０００４】遺伝子療法は費用が高く、一般に利用できないため、別な細胞療法が求められている。分子構造の分析及びタンパク質産生物の機能から、該産生物を産生する遺伝子の保険に関して結論を導くことは可能である。例えば人体の腫瘍抑制機序を決定するためには、多くの場合遺伝子タンパク質産生物を使用して、遺伝子間の相互作用を決定すればよい。腫瘍抑制機序に関しては、本願の親特許出願明細書を参照すればよい。遺伝子機能及び遺伝子相互作用を解明するためには、信頼性が高く、またコストが低い上に、信頼性高くかつ予測可能に多量のタンパク質を提供できる遺伝子タンパク質産生物を産生する方法が非常に望ましい。

【０００５】遺伝子タンパク質産生物は遺伝子機能及び遺伝子相互作用を解明するのに有用なだけでなく、該タンパク質自体が欠損遺伝子状態の治療に使用できる。この場合、欠損遺伝物質をもつ細胞に適当な遺伝子タンパク質産生物を導入することが有利であり、効果的である。場合によって、タンパク質産生物の導入の方が、遺伝物質自体を治療を目的として投与する場合よりもコストが低く、容易なことがある。

【０００６】タンパク質療法については、本願と同時に提出する特許出願明細書を参照すればよい。

【０００７】遺伝子タンパク質産生物の重要性に関する認識からみた場合、実質的に精製された形で遺伝子タンパク質産生物を調製・分離する方法が利用できるようになるのが非常に有利である。生化学的に活性な物質を多量にそのまま利用できることは、遺伝機序に関与している生化学的特性及び分子挙動を研究するためにだけでなく、治療のためにも有意味な前進である。

【０００８】一般に、大量産生の場合と異なる実験室規模の産生では、遺伝子タンパク質産生物は細胞からだけでなく、その合成産生によっても産生している。細胞からの誘導についていえば、細胞タンパク質はごく少量存在しているに過ぎない。この結果、天然源から十分多量なタンパク質を誘導する試みは現実的ではない。

【０００９】合成産生法についていえば、遺伝子の解読配列を細菌発現ベクターに導入してタンパク質を発現する試みは一部の場合を除いて成功していない。細菌産生タンパク質は溶解性が弱い。細菌発現システムを使用する別な欠点は、細菌細胞が真核生物タンパク質を変更できず、またタンパク質について翻訳後変更が必要な場合には、このようなタンパク質の分析が誤った結果を与える点にある。つまり、細菌産生タンパク質は一般に溶解性が低く、分子欠損があるため、有用性に制限がある。

【００１０】タンパク質産生物を作る通常の実験室的方法では、十分に多量のタンパク質を産生できないだけでなく、産生タンパク質の純度も十分でなく、純度にバラツキがある。ある種のタンパク質産生物を産生するさいの難しさの代表例には、ＴｒｐＥ－ＲＢ融合タンパク質を発現し、そしてＥ．ｃｏｌｉに発現するＴ７ＲＮＡポリメラーゼを使用して、ポリペプチドを産生することがある。これら方法は比較的複雑であり、複雑精巧な生化学的操作を必要とする。さらに、これら方法には、目的のポリペプチドがごく少量しか産生しないので、大きな制限がある。また、この方法で産生したポリペプチドは分子的にみて好ましくないことが多い。というのは、例えば、これはホスホリル化しないからである。

【００１１】従って、遺伝子産生物ポリペプチドの重要性から、所望の生化学的及び生物物理学的特性をもつポリペプチドを実質的な量で産生する方法が強く望まれている。

【００１２】生体健康維持における遺伝子の機能の理解については、かなりの進歩がみられる。一般的にいえることは、細胞遺伝子が適正なタンパク質を産生できないと、生体に各種の異常が発生する。この産生できない理由は遺伝子全体が失われているか、あるいは遺伝子自体が各種の原因で欠損していることに求めることができる。これら因子の認識から、遺伝子療法にかなりの進展が認められる。

【００１３】例えば、幼児の腎臓癌であるヴィルムス腫瘍は染色体１１の遺伝子不活性化によって生じると考えられている。一本鎖染色体の微小細胞融合媒介転移法を使用した場合には、正常な染色体１１をヴィルムス腫瘍細胞に導入すると、腫瘍形成性を抑制できることが実証されている。一方、染色体Ｘ及び１３の導入にはこの効果は認められない。

【００１４】実験レベルでは、欠損のないヒト染色体の転移はある程度は有用であるが、このような転移は遺伝子欠損の治療には利用できない。ひとつには、治療に好適な染色体の調製はかなりの熟練を必要とするだけでな

く、時間がかかる上にコストが高いからである。このため、この方法は多くの場合に許容できない。

【００１５】欠損のない染色体を治療に利用するのが好ましくないとした場合、次に理論的に考えられることは、適正な遺伝子全体を、あるいは少なくともその効力のある部分を患者に導入することである。この方法は欠損のない染色体を導入する方法よりも実行可能性が高いが、遺伝子療法が望まれているのはある一定の場合のみである。

【００１６】この点に関して、適正な核酸物質の分離、配列決定、クローン化は非常にコストが高いだけでなく、時間がかかるものである。加えて、このような方法では、世界でもごく限られた場所でしか利用できない複雑精巧な分子遺伝子操作方法が必要である。さらに、現在でも、このような方法は治療に適する多量の物質を産生するようになっていない。

【００１７】以上から、生物工学的方法を利用し、そして比較的コストが低く、信頼性があり、より一般的に利用でき、かつ生化学的作用において特異的な物質を使用する細胞レベルの特定療法が強く望まれている。さらに、細胞レベルで治療に有効な産生物を導入して、腫瘍抑制等の変化をもたらす療法も強く望まれている。いうまでもないことだが、バラツキのない精製状態で多量に産生でき、そして容易にかつ有効に欠損細胞に導入できる産生物についても強く望まれている。

【００１８】本発明の第１目的は癌抑制を制御するのに有効な、全体的に安全でかつ特異的な療法及び産生物を提供することにある。

【００１９】本発明の別な目的は生物工学的方法及び産生物を利用する、癌腫瘍の抑制及び根絶に有効な癌抑制制御産生物及び療法を提供することにある。また、細胞レベル及び細胞間レベルで作用を発揮する、癌治療用薬剤組成物を提供することも本発明の目的である。

【００２０】本発明のさらに別な目的は天然又は合成産生物を活性成分とする、欠損又は突然変異癌抑制遺伝子や、癌抑制遺伝子の不在によって生じる症状を治療する薬剤組成物を提供することにある。

【００２１】本発明は特異的癌抑制遺伝子タンパク質産生物を対象細胞に導き、癌抑制を実現する細胞療法からなる。

【００２２】本発明は従来の放射線療法や化学療法の必要性が少ない、癌の治療法を提供するものである。さらに、本発明方法は遺伝的に癌に罹患しやすい素因の発見後、腫瘍形成が開始する前の極めて早い時期に適用することが可能である。

【００２３】本発明は癌抑制遺伝子タンパク質産生物を好便にかつ比較的低いコストで使用して細胞レベルで癌抑制を実現する点において極めて有利である。

【００２４】ある細胞中のひとつかそれ以上の癌抑制遺伝子が不活性化した場合、それで十分癌を発生することになるかについてははっきりとしていない点があるが、遺伝子タンパク質産生物の細胞導入は新しいアプローチであり、悪性腫瘍治療への有利なアプローチである。本発明は従来からの細胞毒性癌療法とは違い、生体への損傷を最小限に抑えた状態で、細胞レベルで有利な変化をもたらす点においてさらに有利である。

【００２５】上述したように、本発明の第１目的は実質的な量の欠損のない、活性遺伝子産生ポリペプチドを産生する方法を提供することにある。

【００２６】更に本発明の別な目的は構造及び機能が天然産生ポリペプチドと同じである、特異的遺伝子産生ポリペプチドの産生方法を提供することにある。

【００２７】要約すれば、本発明の上記目的及びほかの目的は遺伝物質を昆虫細胞に導入することによって実質的量の所望ポリペプチドを産生する方法により実現できる。例えば、クローン化遺伝子や遺伝子フラグメント又はその誘導体を適当なベクターを利用して、ホスト細胞に導入すれば、高純度のタンパク質を実質的な量でかつ高レベルで産生することができる。

【００２８】本発明は高品質のポリペプチドを実質的量で産生して、細胞レベルで遺伝子機能を調べることできる方法を提供するもので、極めて有利なものである。

【００２９】また、本発明はこのポリペプチドを有利で信頼性があり、しかも反復的に、比較的低いコストで産生できるもので、この点においても非常に有利であり。

【００３０】さらに、本発明は細胞レベル及び細胞間レベルで遺伝子間相互作用を解明できる、相当量の高品質ポリペプチドを産生できるもので、この点でも有利である。

【００３１】

〔図面の簡単な説明〕

本発明の上記上記目的及び特徴、並びにほかの目的及び特徴、そしてこれらの実現方法は以下の記載から明らかになるはずである。また、本発明それ自体の理解は添付図面に参考にして、発明の実施例に関する記載を読めば得られるはずである。図１は$pp110^{RB}$を合成するバキュロウィルス発現ベクターの構成を示す概略図である。図２はｐｐＲＢ感染昆虫細胞のウエスタンブロット（Ｗｅｓｔｅｒｎ　ｂｌｏｔ）である。図３は感染から７２時間後までに感染細胞から抽出した細胞抽出物を示すウエスタンブロットである。図４はＲＢタンパク質の細胞間定位を示す顕微鏡写真である。図５は感染Ｓｆ９細胞の顕微鏡写真である。図６は昆虫細胞におけるＲＢタンパク質のホスホリル化及び脱ホスホリル化分析の結果を示すラジオオートグラフである。図７はｐＭＧ３－２４５抗－ＲＢからの粗溶解産物、感染Ｓｆ９細胞及び溶離物の電気泳動分析結果を示す図である。図８は１０％ＳＤＳ－ＰＡＧＥ、クマシーブリリアントブルー染色法に適用した融合タンパク質及びバキュロウィルス－発現$pp110^{RB}$のサウスウエスタン（Ｓｏｕｔｈｗｅｓ

ｔｅｒｎ）ＤＮＡ結合測定法を示す。図９は図８を作成するために使用したゲルに対する平行ゲルからのブロットのラジオオートグラフであり、このブロットは^{32}Ｐ－標識化ＤＮＡフラグメントでインキュベーションしたものである。図１０バキュロウィルス－発現ＲＢタンパク質とＳＶ４０Ｔ抗原との錯体形成を示すクロマトグラムである。図１１Ｓａｏｓ－２細胞の細胞質への顕微注射後における精製ＲＢタンパク質の核転移を示す。

【００３２】

【発明の最良の実施例】本明細書で言及する文献はいずれもその内容をここに取り入れてあり、本明細書記載の一部を構成している。以下、次の順序で本発明を説明することにする。

Ａ．概説

Ｂ．ＲＢ遺伝子産生物例

Ｂ１．組換えバキュロウィルスの構成

Ｂ２．感染昆虫細胞における外性ＲＢタンパク質の発現

Ｂ３．外性ＲＢタンパク質の核定位及び翻訳後のホスホリル化

Ｂ４．感染昆虫細胞からのＲＢタンパク質精製

Ｂ５．ＤＮＡ一結合活性及びＳＶ４０Ｔ抗原との特異的複合体形成

Ｂ６．精製ＲＢタンパク質の核転移

Ｂ７．要約

【００３３】Ａ．概説

本発明は昆虫細胞培養物に遺伝物質を導入することによって遺伝子タンパク質を産生する方法に関し、この遺伝子タンパク質は培養によって特異的な遺伝子タンパク質産生物を産生するのに使用できる。本方法を使用すれば、力値の高い、実質的に精製された、欠損のない、生化学的に活性なタンパク質を得ることができる。

【００３４】本発明の発現系は広い用途をもつ。すなわち、例えば、ヒトや動物のクローン化遺伝子やそのフラグメント、相同体、誘導体あるいは部分は所望タンパク質産生物の産生に利用することができる。このようにして産生したタンパク質はいうまでもなく欠損細胞の治療に、遺伝子機能の解明に使用できる。といのうは、遺伝子は細胞レベルで相互作用するからである。

【００３５】遺伝子タンパク質産生物を産生する場合、昆虫細胞培養物は真核生物特性をもつので、好適であることが見いだされた。遺伝物質を細胞培養物に導入するためにはウィルスベクター等の通常のベクターを使用することができる。

【００３６】例えば、ＲＢ遺伝子タンパク質産生物を産生するためには、昆虫細胞培養物のウィルスベクターを使用する。この点に関して、培養昆虫細胞において高レベルで組換えタンパク質を産生するためには、ヘルパーに依存しないウィルス発現ベクターとしてバキュロウィルスのオートグラファ・カリホルニカ（Ａｕｔｏｇｒａｐｈａ　ｃａｌｉｆｏｒｎｉｃａ）、即ち核多角体病ウィルス（ＡｃＮＰＶ）を使用する。このウィルスは培養したアメリカ産行列毛虫ヨトウガ、即ちスポドプテラ・フルギペルダ（Ｓｐｏｄｏｐｔｅｒａ　ｆｒｕｇｉｐｅｒｄａ）（Ｓｆ９）細胞中で繁殖する。このウィルスは多角体遺伝子の強力な、一時的に調節されたプロモーターをもつものであり、その産生物は溶解感染時に全細胞タンパク質の５０％以上を占める。インビボ組換えにより、外来遺伝子の解読配列はポリヘドリンプロモーターの翻訳制御を受け、この結果高レベルでタンパク質を発現する。さらに、このようにして産生したタンパク質は正確に倍加し、元の高級真核生物に見られるタンパク質と同様な翻訳後変更を含んでいる。

【００３７】本発明は特異的な癌抑制遺伝子タンパク質産生物を対象細胞に導入して、腫瘍抑制を実現する細胞療法である。所与の細胞が、欠損遺伝子をもつため、細胞におけるタンパク質発現欠損を招く点において欠損していることがある。

【００３８】本発明方法は欠損遺伝子に関係する遺伝子タンパク質産生物を使用することに関する。精製タンパク質産生物を対象細胞に導入して、例えば、腫瘍抑制を実現する。この産生物を薬理学的に好適な担体に担持して導入することにより、該タンパク質産生物が細胞レベルで、あるいは細胞下レベルで作用する。

【００３９】この方法の一例として、癌抑制遺伝子であるＲＢ遺伝子が欠損しているか、あるいはそれがない細胞ＲＢ遺伝子タンパク質をに導入した。ＲＢ遺伝子に関する詳しい情報については、本明細書で言及している親特許出願明細書を参照すればよい。

【００４０】好適な実施例では、劣性腫瘍形成研究のプロトタイプモデルとして網膜芽腫、即ち発育中の網膜の稀な症状である、幼児癌を使用する。染色体１３ｑ１４に対する、関与している遺伝要素の定位及びその劣性性に基づき、癌抑制遺伝子と推定されている遺伝子、即ち網膜芽腫感受性遺伝子（ＲＢ）をクローン化した。この遺伝子は２７のエクソンが２００キロ塩基のゲノムＤＮＡに分散し、検査した正常な組織にすべてに４．７キロ塩基ｍＲＮＡ転写を発現する。相補ＤＮＡクローンの配列分析により、９２８のアミノ酸の仮説的タンパク質をコード化できた長いオープンリーディングフレームが明らかになった。ＲＢｃＤＡＮ配列から推測した所定エピトープに対する抗体を使用したところ、このＲＢ遺伝子産生物は相対分子量（Ｍｒ）が１１０，０００～１１４，０００の核リンタンパク質と同定された。これを$p110^{RB}$と命名した。

【００４１】網膜芽腫にだけではなく、ＲＢ遺伝子機能の欠損はまた乳癌、骨肉腫、前立腺癌や小細胞肺癌を始めとする他の幾つかの腫瘍にもに関与していることが判明している。最近の研究によれば、レトロウィルス媒介遺伝子転移により網膜芽腫、骨肉腫や前立腺癌細胞にＲＢ遺伝子を再導入すると、ヌードマウスにおける腫瘍形

成を始めとする新生表現型の幾つかの面を明らかに抑制することが判明している。これはＲＢ遺伝子の腫瘍抑制機能の直接的な証拠である。ところが、この生物学的活性の分子レベルにおける基礎はまだ確立されていない。

【００４２】現在までのところ、癌抑制遺伝子産生物の生化学的特性及び生物学的機能はいずれも解明されていないが、これはこのタンパク質が細胞中に少ないため、十分な量で得ることが難しいからである。この点に関しては、本願と同時に出願した親出願明細書（発明の名称：遺伝子タンパク質産生物の産生方法）を参照すればよい。

【００４３】欠損遺伝子をもつ、あるいは遺伝子のない細胞に特異的遺伝子タンパク質産生物を導入する細胞療法を発明した。本発明によれば、実質的に精製された、欠損のない、生化学的に活性な遺伝子産生タンパク質の適正な量を治療上有効な投与量で欠損遺伝子に導入できる。

【００４４】本発明の細胞療法は適用用途が広い。本タンパク質産生物は欠損細胞の治療だけでなく、遺伝子機能の解明にも有用である。というのは、遺伝子が細胞レベルで相互作用するからである。

【００４５】ひとつの特定実施例では、網膜芽腫遺伝子タンパク質産生物、即ちｐｐ１１０^{RB}は欠損遺伝子をもつ、あるいは遺伝子のない真核生物細胞の治療に広く適用できる。

【００４６】なお、本発明によれば、真正なヒトｐｐ１１０^{RB}と全く同様に、精製タンパク質がＤＮＡを結合し、ＳＶ４０Ｔ抗原と特異的錯体を形成できた。また、顕微注射後直ちに行ったタンパク質の核転移によると、このタンパク質が活性を示し、治療に使用できることが判明した。

【００４７】Ｂ．ＲＢ遺伝子産生例

次に、ｐｐ１１０^{RB}タンパク質の本発明による産生方法についてさらに詳しく説明する。このタンパク質及び網膜芽腫遺伝子に関するさらに詳細な情報については、以下の親特許出願明細書を参照すればよい。

【００４８】ＲＢ遺伝子等の癌抑制遺伝子産生物の生化学的特性及び生物学的機能はいずれも解明されていないが、これは精製タンパク質を十分な量で得ることが難しいからである。即ち、ひとつには、タンパク質が細胞に少ないからであり、また加えて、遺伝子のコード化配列の細菌発現ベクターへの導入によるタンパク質発現が一部を除いて成功していないからである。これらから、現在利用されている方法がもつ問題は真核生物系にクローン化遺伝子を発現すれば避けることができると結論できた。本発明の特定実施例はＲＢ遺伝子タンパク質産生物の産生に関するが、本発明は、例示すれば癌抑制遺伝子であるが、とはいってもこれに制限されない他の真核生物遺伝子のタンパク質産生物の産生にも適用でき、従ってこのようなタンパク質産生物の産生にも関するものである。

【００４９】培養昆虫細胞において高レベルで組換タンパク質を産生する為には、ヘルパーに依存しないウィルス発現ベクターとしてバキュロウィルスのオートグラファ・カリホルニカ（Ａｕｔｏｇｒａｐｈａ　ｃａｌｉｆｏｒｎｉｃａ）、即ち核多角体病ウィルス（ＡｃＮＰＶ）を使用するが好ましい。このウィルスは培養したアメリカ産行列毛虫ヨトウガ、即ちスポドプテラ・フルギペルダ（Ｓｐｏｄｏｐｔｅｒａ　ｆｒｕｇｉｐｅｒｄａ）（Ｓｆ９）細胞中で繁殖する。このウィルスは多角体遺伝子の強力な、一時的に調節されたプロモーターをもつものであり、その産生物は溶菌感染時に全細胞タンパク質の５０％以上を占める。インビボ組換により、外来遺伝子の解読配列はポリヘドリンプロモーターの翻訳制御を受け、この結果高レベルでタンパク質を発現する。さらに、このようにして産生したタンパク質は正確に倍加し、元の高級真核生物に見られるタンパク質と同様な翻訳後変更を含んでいる。

【００５０】バキュロウィルス系により機能性ＲＢタンパク質の発現可能性をテストする為に、ＲＢ遺伝素の完全なコード化配列を含むクローン化ヒトＲＢｃＤＮＡをＡｃＮＰＶ発現ベクターに導入し組換えウィルスを昆虫細胞中で繁殖させた。ホストーベクター系により高レベルでヒトｐｐ１１０^{RB}発現を実現できた。得られたタンパク質をホスホリル化し、感染細胞の核を正確に標的とした。さらに、ＲＢタンパク質の精製法も確立した。なお、本発明によれば、真正なヒトｐｐ１１０^{RB}と全く同様に、精製タンパク質がＤＮＡを結合し、ＳＶ４０Ｔ抗原と特異的複合体を形成できた。また、顕微注射後直ちに行ったタンパク質の核転移によると、このタンパク質が活性を示し、治療に使用できることが判明した。

【００５１】Ｂ１．組換えバキュロウィルスの構成

バキュロウィルス発現系においてＲＢタンパク質を最大限に産生するために、ＲＢ遺伝子から大部分の５’非コード化配列を取り除くことにより組換え転移ベクターを構成した。座特異性突然変異誘発によって、ＲＢｃＤＡＮのヌクレチオド１１６及び２９３５に２つのＢａｍＨ１座を導入して、組換え転移ベクターの構成を促進した。図１に示すように、精製したｐＡｃＹＭ１／ＲＢ２．８は、完全な（６０塩基対）ポリヘドリン５’非コード化配列がＲＢｃＤＮＡの５’未翻訳領域に融合し、その後に完全なコード化配列が続くｍＲＡＮをコード化する。この組換え遺伝子は真正なＲＢ開始座のヌクレチオド１３９の上流にＡＴＧコドンを含まない。従って、組換え遺伝子は非融合、フルレングスＲＢタンパク質をコード化する。

【００５２】図１に転移ベクターｐＡｃＹＭ１を示すが、これらはすべて開始ＡＴＧコドンのＡを含むポリヘドリン遺伝子の上流配列をもち、この後に特徴的なＢａＭＨ１座が続く。転移ベクターについては、松浦等の記

載がある。Ｊ．Ｇｅｎ．Ｖｉｒｏｌ．，６８：１２３３－１２５０（１９８７）。ｐＲＢ４４－２はプラズマｐＧＥＭ１（Ｐｒｏｍｅｇａ）のＢａｍＨ１座にサブクローン化したヌクレオチド１１６から２９３５にいたる完全なＲＢｃＤＮＡコード化配列を含む。組換えバキュロウィルスベクター、即ちｐＡｃＹＭ１／ＲＢ２．８については、２．８ｋｂのＢａｍＨ１フラグメントをｐＲＢ４４－２から適正な配向方向にｐＡｃＹＭ１のＢａｍＨ１座に挿入して構成したため、ＲＢ遺伝子の転写をポリヘドリンプロモーターの直接制御下におくことができた。

【００５３】バキュロウィルス発現ベクターを構成してｐｐ１１０^{RB}を合成するさいには、以下の点を考慮した。ｐ^{RB}４４－２はｐＧＥＭ１のＢａｍＨ１座にサブクローン化したヌクレオチド１１６から２９３５にいたる完全なＲＢｃＤＮＡコード化配列からなる。ｐＡｃＹＭ１はポリヘドリン遺伝子の側面に位置するウィルスＤＮＡ配列の約７ｋｂのＥｃｏＲ１フラグメントを含み、この場合先導配列はそのままであるが、ＡＴＧの第１Ａを除くポリヘドリンコード化配列はすべてＢａｍＨ１リンカーによって置換する。ポリヘドリンプロモーターＲＢｃＤＮＡ融合を含む組換えバキュロウィルスベクター、すなわちｐＡｃＹＭ１／ＲＢ２．８については、２．８ｋｂのＢａｍＨ１フラグメントをｐＲＢ４４－２から適正な配向方向にｐＡｃＹＭ１のＢａｍＨ１座に挿入して構成したため、ＲＢ遺伝子の転写をポリヘドリンプロモーターの直接制御下におくことができた。図１の下の方に、融合接合部の配列を示す。また、図１の下のケース記号はポリヘドリンプロモーターを、上のケース記号はＲＢｃＤＮＡ配列を示す。そしてＢａｍＨ１リンカーにはアンダーラインしてある。ＲＢのＡＴＧ（ヌクレオチド１３９）を利用する融合遺伝子の翻訳は矢印で示す。図１のａ＊（＋１）はポリヘドリン遺伝子の翻訳開始コドンＡＴＧの第１Ａを示す。

【００５４】ＲＢｃＤＮＡの組換えプラスミドからウィルスゲノムへの転写は、リポフェクション（ｌｉｐｏｆｅｃｔｉｏｎ）により野生型Ａｕｔｏｇｒａｐｈａ ｃａｌｉｆｏｒｎｉｃａ核多角体病ウィルスＤＮＡを用いてＰａＣｙｍ１／ＲＢ２．８ＤＮＡをコントランスフェクション（ｃｏｎｔｒａｎｓｆｅｃｔｉｏｎ）することにより行った（ＢＲＬ）。同族組換えによりＲＢ遺伝子とのアレリック置換によりポリヘドリン遺伝子を不活性化した組換えウィルスは、感染細胞にポリヘドリン閉鎖体が認められなかったので、そのプラーク形態によって同定した。ウィルスを３回プラーク精製して、純粋なバキュロウィルス株を得た。これをＡｃＮＰＶ－Ｙ４ＲＢとした。

【００５５】Ｂ２．感染昆虫細胞における外生ＲＢタンパク質の発現

ＡｃＮＰＶポリヘドリンプロモーターが異種無脊椎動物細胞にヒトＲＢ遺伝子の発現をもたらすかどうかを決定する前に、Ｓｆ９細胞を用意し、３．３３ｇｍ／ｌのイーストレート（ｙｅａｓｔｏｌａｔｅ）、ラクトアルブミン加水分解産生物（ＧＩＢＣＯ）及び１０％の加熱不活性化ウシ胎児血清（ＧＩＭＩＮＩ社）を加えたグレースの昆虫培地（Ｇｒａｃｅ’ｓ ｉｎｓｅｃｔ ｍｅｄｉｕｍ）－Ｂｕｌｌ．１５５５，（１９８７）（テキサス農業実験所、カレッジ実験所、ＴＸ）を用いて、２７℃で単層又は懸濁培養によりＳｆ９、即ちＳｐｏｄｏｐｔｅｒａ ｆｒｕｇｉｐｅｒｄａ ＩＰＬＢ－Ｓｆ２１－ＡＥ［Ｉｎ ｖｉｔｒｏ，１３：２１３－２１７（１９７７）］を培養した。細胞溶解産物を大規模産生するさいには、Ｓｆ９細胞のスピンナー培養物を血清を含まないＥＸ－ＣＥＬＬ４００規定培地（Ｊ．Ｒ．Ｓｃｉｅｎｔｉｆｉｃ社）で培養した。ヒトＴ白血病細胞系統のモルト（Ｍｏｌｔ）－４細胞を２０％子ウシ血清を加えたＲＰＭＩ１６４０培地で懸濁培養した。骨肉腫細胞系統のＳａｏｓ－２細胞を７．５％ウシ胎児血清を加えたダルベッコ（Ｄｕｌｂｅｃｃｏ）変性イーグル培地で培養した。

【００５６】ＡｃＮＰＶポリヘドリンプローモータが異種無脊椎動物細胞にヒトＲＢ遺伝子の発現をもたらすかどうかを決定するさいには、プラーク精製ＡｃＮＰＶ－Ｙ４ＲＢでＳｆ９細胞を感染処理した。感染４０時間後に、感染細胞の溶解産物を集め、抗－ＲＢＯ－４７抗原で免疫沈降処理した。次に、サンプルをＳＤＳ－ＰＡＧＥ処理してから、ウエスタンブロット分析した。

【００５７】図２及び図３に、ウエスタンブロット分析により行ったＡｃＮＰＶ－Ｙ４ＲＢ感染昆虫細胞におけるｐｐ１１０^{RB}同定結果を示す。図２の場合、感染４０時間後に、模擬感染細胞（経路２）、ＡｃＮＰＶ－Ｙ４ＲＢ感染細胞（経路３）、あるいは野生型ＡｃＮＰＶ感染Ｓｆ９細胞（経路４）から細胞抽出物を調製した。ヒト白血病細胞系統のモルト－４を対照として使用した（経路１）。

【００５８】図３に示すように、単クローン系抗体で免疫ブロット処理した場合、ＡｃＮＰＶ－Ｙ４ＲＢ感染細胞の抽出物（経路３）においては哺乳動物細胞（経路１）と同様のフルレングスのＲＢタンパク質を発現が認められたが、模擬感染細胞や野生型ＡｃＮＰＶ感染細胞（経路２、４）の場合には認められなかった。図３の場合には、ＲＢタンパク質産生の最適時期を求めるために、感染後時間をかえて、ＡｃＮＰＶ－Ｙ４感染細胞から細胞抽出物を得た。溶解産物を抗－ＲＢＯ．４７抗原で免疫沈降処理し、ｐＭＧ３－２４５単クローン系抗体で免疫ブロット処理した。図３のｐ１１０^{RB}及びｐｐ１１０^{RB}はそれぞれ未ホスホリル化タンパク質及びホスホリル化タンパク質を表す。感染後は、ＲＢタンパク質の産生をモニターして、細胞を回収する最適な時期を求めた。図３に示すように、ＲＢタンパク質産生は感染２４

時間後に検出でき、続く１２時間の間に産生量はかなり増加した。タンパク質産生レベルは感染後約７２時間にわたりずっと維持し、そして７２時間後に細胞の溶菌が実質的に開始した。細胞溶解に伴うタンパク質劣化を最小限に抑えるために、通常、感染から約４０時間後に、感染細胞を回収した。

【００５９】ＲＢタンパク質の発現を検出する際には、ＡｃＮＰＶ－Ｙ４ＲＢを使用して、０．５のＭＯＩでＳｆ９細胞を感染処理した。感染からそれぞれ２４、３６、４８、６０及び７２時間後に、５ｘ１０4の細胞を１ｍｌの溶解緩衝液（５０ｍＭのＮａＣｌ、ｐＨ７．４；０．２％のノニデット（Ｎｏｎｉｄｅｔ）Ｐ－４０；１ｍＭのＥＤＴＡ；１００ｍＭのＮａＣｌ；５０ｍＭのＮａｆ及び１ｍＭのＰＭＳＦ）中で溶解し、溶解産物を５分間遠心処理して清澄化した。溶解産物を次に抗－ＲＢＯ．４７抗原でインキュベーションして、免疫沈降物を７．５％ＳＤＳ－ＰＡＧＥで分離した。この後、タンパク質をニトロセルロース紙に移し、常法で処理した。一夜遮断状態で放置した後、３時間ニトロセルロース紙をｐＭＧ３－２４５抗－ｆＲＢ単クローン系抗原でインキュベーション処理してから、Ｃｅｌｌ、５４：２７５－２８３（１９８８）の記載に従ってアルカリ性ホスファターゼ接合ヤギ抗－マウスＩｇＧ及び発色基質で処理した。

【００６０】Ｂ３．外生ＲＢタンパク質の核定位及び翻訳後のホスホリル化

ＲＢ遺伝子は分子量（Ｍｒ）が１１０，０００の核リンタンパク質をコード化する。バキュロウィルスにより昆虫細胞に産生したＲＢタンパク質が核に正確に到達したかを調べるために、感染から４０時間後に抗－ＲＢＯ．４７抗原を用いてＡｃＮＰＶ－Ｙ４ＲＢ－感染Ｓｆ９細胞を免疫染色した。免疫染色により昆虫細胞に発現したＲＢタンパク質の細胞間定位を図４及び図５に示す。図４には模擬感染Ｓｆ９細胞の場合を、そして図５にＡｃＮＰＶ－Ｙ４ＲＢ－感染Ｓｆ９細胞の場合を示す。図４及び図５に示すように、感染細胞は異常に大きい核を含んでいた。このような状態はバキュロウィルス感染の細胞変性効果に特有なものである。模擬感染又は野生型ＡｃＮＰＶ感染Ｓｆ９細胞を抗－ＲＢＯ．４７抗原でインキュベーションしたところ、染色は認められなかった（図４）。ところが、ＡｃＮＰＶ－Ｙ４ＲＢ感染細胞の核にのみ強い染色が認められた（図５）。ＡｃＮＰＶ－Ｙ４ＲＢ感染Ｓｆ９細胞からの核及び細胞形質抽出物をＳＤＳ－ＰＡＧＥ及びウエスタンブロッティングにより分析したところ、外生ＲＢタンパク質は主に核部分に存在することが確認できた。

【００６１】免疫染色は次の工程で行った。模擬感染、野生型ＡｃＮＰＶ感染、ＡｃＮＰＶ－Ｙ４感染のいずれも場合も感染から４０時間後にＳｆ９細胞をポリ－Ｌ－リシン（Ｓｉｇｍａ社）をコーチングしたチャンバースライド（Ｍｉｌｅｓ　Ｓｃｉｅｎｔｉｆｉｃ社）に接種し、一夜インキュベーションした。いずれも次の工程に移る前にスライドをリン酸緩衝塩化ナトリウム水溶液で洗浄した。即ち、まず４％ホルムアルデヒドの０．０４Ｍリン酸緩衝液を用いて２０分間、あるいはアセトン（－２０℃）を用いて１０分間細胞を固定した。２％の正常なヤギ血清のＰＢＳ溶液で１０分間固定細胞を前インキュベーションしてから、０．０２％のトリトンＸ－１００に希釈した抗－ＲＢＯ．４７抗原で一夜インキュベーションした。洗浄後、ビオチニル化したヤギ抗－ウサギＩｇＧ（カナダ、バーリンゲームのＴＡＧＯ社）を加えた。一時間後、セイヨウワサビペルオキシダーゼ（カナダ、バーリンゲームのＶｅｃｔｏｒ　Ｌａｂｓ社）で接合したＡＢ複合体を用いて４５分間細胞をインキュベーションしてから、次の基質を用いてインキュベーションした。この基質は０．０５％の３，３’－ジアミノベンジジンテトラヒドロクロリド、及び０．０１％のＨ2Ｏ2の０．０５Ｍトリス－ＨＣｌ溶液から構成した。ｐＨ７．６（Ｓｉｇｍａ社）。３～５分後に細胞をＰＢＳ洗浄することにより反応を停止した。次に、ニコン製のダイアフォトマイクロスコープ（ｄｉａｐｈｏｔｏｍｉｃｒｏｓｃｏｐｅ）で細胞の写真を取った。

【００６２】図６に、昆虫細胞に産生したＲＢタンパク質のホスホリル化及び脱ホスホリル化の結果を示す。ＡｃＮＰＶ－Ｙ４ＲＢ感染から４０時間後に、35 Ｓ－メチオニンあるいは32 Ｐ－オルトリン酸を用いて３時間でＳｆ９細胞を代謝標識化した。対照としてモルト－４を使用し、抗－ＲＢＯ．４７抗原を用いて細胞溶解産物を免疫沈降した。ポテト酸ホスファターゼ（ＰＡＰ）処理する前に（経路１、２、３、４）、あるいは処理後に（経路１’、２’、３’、４’）ＳＤＳ－ＰＡＧＥにより35 Ｓ－及び32 Ｐ－標識化ＲＢタンパク質免疫複合体を分離し、ラジオオートグラフィーにより分析した。同様に、未標識化細胞からの溶解産物を使用して脱ホスホリル化実験を行い、ポテト酸ホスファターゼ処理前後（経路５、６及び５’、６’）にウエスタンブロット分析を行った。

【００６３】さらに続けて図４の場合を説明する。ＲＢタンパク質のホスホリル化は多重セリン及びトレオニン残基で生じるもので、ＳＤＳ－ＰＡＧＥにおけるＲＢタンパク質の分子量不均一性を説明するものである。［Ｏｎｃｏｇｅｎｅ　Ｒｅｓ．，１：２０５－２１４（１９８９）；Ｃｅｌｌ，５６：５７－６５（１９８９）］昆虫細胞に産生したＲＢタンパク質が翻訳後にホスホリル化を受けるかどうかを調べるために、感染後３時間から４０時間で35Ｓ－メチオニンあるいは32Ｐ－オルトリン酸を用いてＡｃＮＰＶ－Ｙ４ＲＢ－感染Ｓｆ９細胞を代謝標識化した。細胞抽出物を免疫沈降処理し、ＳＤＳ－ＰＡＧＥ分析してから、ラジオオートグラフィー処理した。この点については、図６の経路２及び４をそれぞれ

参照すればよい。これと平行して、同じ抽出物からの免疫沈降性ＲＢタンパク質をポテト酸ホスファターゼ（ＰＡＰ）で処理して、ＳＤＳ－ＰＡＧＥにおけるＲＢタンパク質移動性への脱ホスホリル化効果をテストした。脱ホスホリル化後、^{35}Ｓ－標識化ＲＢタンパク質の分子量１１０，０００は２重バンドから１重バンドに落ちた（図６の経路２’）。そして^{32}Ｐ－標識化ＲＢタンパク質から殆ど完全に放射能が放出した（図６の経路４’）。ＡｃＮＰＶ－Ｙ４ＲＢで感染処理した、未標識化細胞からの溶解産物についてのウエスタンブロッティングによる脱ホスホリル化分析も、ＰＡＰ処理と同じバンド変化パターンを示した（図６の経路６、６’）。これら観察結果から、昆虫細胞に産生したＲＢタンパク質はホスホリル化していたことがわかった。また、この変化からＳＤＳ－ＰＡＧＥで認められたＲＢタンパク質の分子量不均一性を説明できた。

【００６４】Ｓｆ９昆虫細胞の放射線標識化及び脱ホスホリル化分析は次に示す工程で実施した。感染から４０時間後に、１０％のウシ胎児血清を加えた、メチオニン、リン酸のいずれも含まないＤＭＥ培地で６０ｍｍの皿を用いて３０分間Ｓｆ９細胞（３ｘ１０^{6}）をインキュベーションした。この後、３時間で０．２５ｍＣｉ／ｍｌの^{35}Ｓ－メチオニン（１１３４Ｃｉ／ｍｍｏｌｅ、ＮＥＮ）か０．２５ｍＣｉ／ｍｌの^{32}Ｐ－オルトリン酸（担体なし、ＩＣＮ）のいずれかを加えることにより細胞を代謝標識化した。溶解緩衝液（５０ｍＭのトリス－ＨＣｌ、ｐＨ７．４；０．２％のノニデットＰ－４０；１ｍＭのＥＤＴＡ；１００ｍＭのＮａＣｌ；５０ｍＭのＮａＦ及び１ｍＭのＰＭＳＦ）を用いて細胞を抽出し、抽出物を抗－ＲＢ０．４７抗原で免疫沈降処理した。

【００６５】^{35}Ｓ－又は^{32}Ｐ－標識化細胞溶解産物、及び未標識化細胞溶解産物からの免疫沈降ＲＢタンパク質の２／３をポテト酸ホスファターゼ（ＰＡＰ、Ｂｏｅｈｒｉｎｇｅｒ社）脱ホスホリル化分析した。［Ｏｎｃｏｇｅｎｅ　Ｒｅｓ．、１：２０５－２１４（１９８９）］。反応緩衝液（２０ｍＭのＭＥＳ、ｐＨ５．５；１００ｍＭのＮａＣｌ；１ｍＭのＭｇＣｌ２；５０μＭのロイペプチン）３７℃、６０分間で１．５単位のＰＡＰを用いて、ＲＢタンパク質含有免疫複合体をインキュベーションし、反応後、ＲＢタンパク質を７．５％ＳＤＳ－ＰＡＧＥ分析してから、ラジオオートグラフィーかウエスタンブロッティングにより分析した。

【００６６】Ｂ４．感染昆虫細胞からのＲＢタンパク質の精製

１．０の感染多重度（ＭＯＩ）でＡｃＮＰＶ－Ｙ４ＲＢによりＳｆ９細胞を感染処理し、感染から４０時間後に細胞溶解産物を得た。この条件でバキュロウィルス系に発現したＲＢタンパク質の全レベルは感染昆虫細胞培養物１リットルにつき約１７～１８ｍｇであった（”１０^{9}細胞）。この点については、表１を参照すれべよい。

【００６７】

【表１】

表１

バキュロウィルス感染昆虫細胞からの組換えＲＢタンパク質の精製

工程	合計タンパク質量（ｍｇ）	ＲＢタンパク質量（ｍｇ）	収率（％）	精製度	純度（％）
細胞抽出	６７０[a]	１６[c]	９０[c]	１．０倍	２．３
ｐＭＧ３－２４５免疫親和カラム	１３．５[b]	１２．９[d]	７２[cd]	４１．３倍	９５

ａ．ブラッドフォード（Ｂｒａｄｆｏｒｄ）（Ｂｉｏ－ＲＡＤ社）の方法によるタンパク質定量

ｂ．マイクロＢＣＡ（ＰＩＥＲＣＥ社）及び分光測光法によるタンパク質定量

ｃ．ウエスタンブロット法及び濃度測定法によるタンパク質定量

ｄ．クマシーブリリアントブルー染色法及び濃度測定法によるタンパク質定量

【００６８】表１に示すように、発現したＲＢタンパク質の９０％（１６ｍｇ）は細胞分裂後の上澄みに認められ、１０％が不溶画分に止まっていた。全細胞タンパク質の２．３％を占めていたので、ＲＢタンパク質は細胞溶解産物から簡単に検出できた。１段免疫親和クロマトグラフィー精製後、約１３５ｍｇのタンパク質をカラムのアルカリ性溶離物から回収できた。溶離ＲＢタンパク質の純度を調べるために、２．５ｘ１０^{5}細胞に相当する溶離物アリコットをＳＤＳ－ＰＡＧＥ及びクマシーブリリアントブルー染色により分析した。

【００６９】この点に関しては、ｐｐ１１０^{RB}の免疫親和クロマトグラフィー精製を示す図７を参照すればよい。１ｘ１０^{5}の、模擬感染Ｓｆ９細胞（経路１）又はＡｃＮＰＶ－Ｙ４ＲＢ感染Ｓｆ９細胞（経路２）からの粗溶解産物、及びｐＭＧ３－２４５抗－ＲＢ免疫親和クロマトグラフィーから溶離物のアリコット（２．５ｘ１０5感染細胞に相当）を１０％ＳＤＳ－ポリアクリロアミドゲルによる電気泳動法によって分析してから、クマシーブリリアントブルー染色法により分析した。予想分子量のＲＢタンパク質は矢印で示してある。

【００７０】濃度測定法により判断すると、上記１段精製工程は有効であり、ＲＢタンパク質を純度９５％（図７、経路３）、収率７２％、そして精製度４１．３倍で得ることができた（表１）。

【００７１】免疫親和カラムはシュナイダー等や島西等の記載に従って構成したが、若干の変更を加えた。［Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．、２５７：１０７６６－１０７６９（１９８２）；Ｖｉｒｏｌｏｇｙ、１４４：８８－１００（１９８５）］。バイオラッド（Ｂｉｏ－Ｒａｄ）カラムに２ｍｌのタンパク質Ｇ－アガロース（Ｇｅｎｅｘ社）を充填し、０．０１ＮのＨＣｌで洗浄した後、結合緩衝液（０．１Ｍの酢酸ナトリウム、ｐＨ５．０；０．１ＭのＮａＣｌ）で洗浄した。カラムに２回１５ｍｇの抗－ｆＲＢ単クローン性抗原（ｐＭＧ３－２４５）を加え、結合を行った。次に、カラムを０．１Ｍの硼酸緩衝液で広く洗浄し、ビーズを２０ｍｌの緩衝液に再懸濁した。最終濃度が４０ｍＭになるようにジメチルピメリミデートジヒドロクロリド（Ｓｉｇｍａ社）を加え、混合物を室温で１時間攪拌し、架橋反応を行った。洗浄後、室温で１０分間、４０ｍＭのエタノールアミン－ＨＣｌの０．１Ｍ硼酸緩衝液２０ｍｌ溶液、ｐＨ８．０、でビーズの残留反応性基を遮断した。この後、０．２Ｍのグリシン、ｐＨ２．３、でカラムを洗浄し、トリス緩衝液（５０ｍＭのトリス－ＨＣｌ、ｐＨ７．４；１００ｍＭのＮａＣｌ；１ｍＭのＰＭＳＦ：１ｍＭのＥＤＴＡ）で中和し、必要になるまでこの溶液に保存した。最初の単クローン性抗原サンプルのOD^{280} 及び次の工程における流動画分のＯＤ280を測定したところ、約１０ｍｇのｐＭＧ３－２４５が２ｍｌのタンパク質Ｇ－アガロースに結合していた。

【００７２】Ｂ５．ＤＮＡ結合活性及びＳＶ４０Ｔ抗原との特異的複合体形成

現在のところ、ＲＢタンパク質については２つの生化学的特性が記述されている。即ち、固有な性質としてＤＮＡを結合できることがそのひとつであり［Ｎａｔｕｒｅ、３２９：６４２－６４５（１９８７）］、そして何種類かのＤＮＡ腫瘍ウィルスの腫瘍タンパク質と特異的複合体を形成できることがもうひとつである［Ｃｅｌｌ、５４：２７５－２８３、（１９８８）；Ｓｃｉｅｎｃｅ、２４３：９３４－９３７（１９８９）；Ｎａｔｕｒｅ（Ｌｏｎｄｏｎ）、３３４：１２４－１２９（１９８８）］。タンパク質の生物学的機能に関与しているこれら２つの公知生化学的特性について、バキュロウィルス感染昆虫細胞から精製したＲＢタンパク質をテストした。

【００７３】図８及び図９にサウスウエスタンＤＮＡ－結合測定法を示す。６μｇの精製ｔｒｐＥ－ＲＢ融合タンパク質、及び精製バキュロウィルス発現$pp110^{RB}$を１０％ＳＤＳ－ＰＡＧＥに適用した。図８に示した結合測定法の場合には、クマシーブリリアントブルー染色法を使用し、図９に示した結合測定法の場合には、ニトロセルロース紙に平行ゲルを電気転写した。ブロットを次に^{32}Ｐ－標識化ＤＮＡ画分でインキュベーションし、ラジオオートグラフィー分析した。図８及び図９の場合次の通りである。経路１：ＲＢ１９－２２；経路：ＲＢ２３－２７；経路３：ＲＢ１９－２７；経路４：ＡｃＮＰＶ－Ｙ４ＲＢ感染昆虫細胞からの精製ＲＢタンパク質。

【００７４】図８及び図９の場合、ＤＮＡ結合をサウスウエスタン分析により測定した。この場合、同量のｔｒｐＥ－ＲＢ融合タンパク質、及び昆虫細胞からの精製ＲＢタンパク質を１０％ＳＤＳ－ＰＡＧＥにより分離した。ローディングされたタンパク質の量はクマシーブリリアントブルー染色法によって求めた（図８）。別な平行ゲルを使用した実験では、ニトロセルロース膜に電気転写してから、^{32}Ｐ－標識化ＤＮＡでインキュベーションした。タンパク質に結合したＤＮＡを次にラジオオートグラフィーにより分析した（図９）。その主ドメインがＤＮＡと相互作用する融合タンパク質ＲＢ１９－２７のＤＮＡに対する親和力は２つの領域ＲＢ１９－２２及びＲＢ２３－２７よりも２０倍高い。この点については、図９の経路３は経路１及び２に比較でき、精製フルレングスＲＢタンパク質はＲＢ１９－２７と同様に強いＤＮＡ結合活性を示した（図９、経路４）。昆虫細胞からの精製ＲＢタンパク質のＤＮＡ結合活性はＤＮＡセルロースによるタンパク質の保持によっても、またカラムからのその後に続く溶離－約４００ｍＭのＮａＣｌ－によっても証明できた。

【００７５】感染昆虫細胞からの$pp100^{RB}$の精製は次のようにして実施した。１．０のＭＯＩでＡｃＮＰＶ－Ｙ４ＲＢを用いてＳｆ９細胞を感染処理し、懸濁培養した（１Ｘ16^{6}細胞／ｍｌ、１０００ｍｌ）。感染から４０時間後に、低速遠心分離によって細胞をペレット化し、洗浄し、そして５０ｍＭのトリス－ＨＣｌ、ｐＨ７．４；０．２％のＮＰ－４０；１ｍＭのＥＤＴＡ；１００ｍＭのＮａＣｌ；１０％の（ｖ／ｖ）グリセロール；１ｍＭのＤＴＴ；１ｍＭのＰＭＳＦ；２５μｇのロイペプチン及び５０単位／ｍｌのアプロチニンを含む抽出緩衝液に再懸濁した。氷上で１５分間インキュベーションした後、サンプルを遠心（１０，０００ｘｇ、４℃、１０分間）清澄化処理し、ＲＢ含有上澄みを回収した。前述したように、タンパク質Ｇ－アガロースに結合した抗－ｆＲＢ単クローン性抗原（ｐＭＧ３－２４５）を含む２ｍｌ容量のカラムで$pp110^{RB}$の免疫親和クロマトグラフィーを実施した。上澄みをカラムに４回通した後、それぞれ次の溶液を２００ベッド容量の量で用いてカラムを連続的に洗浄した。溶解緩衝液、５００ｍＭのＮａＣｌを含む溶解緩衝液及び洗浄液（２００ｍＭのＮａＣｌ；１ｍＭのＥＤＴＡ；１ｍＭのＤＴＴ；１ｍＭのＰＭＳＦ；１０％のグリセロール）。次に、２ｍＭのトリエチルアミン、ｐＨ１０．８；２００ｍＭのＮａＣｌ；１ｍＭのＥＤＴＡ；１ｍＭのＰＭＳＦ及び１０％のグリセロールを含むアルカリ性溶離緩衝液で結合タンパク質をカラムから溶離した。１ｍｌ画分を回収し、直

ちに１Ｍのトリス－ＨＣｌ（ｐＨ７．５）の１／１２容量で中和し、１０％グリセロール中に－７０℃で保存した。

【００７６】感染昆虫細胞からｐｐ１１０^{RB}を精製するさいに、全タンパク質量を求めてから、サウスウエスタンＤＮＡ結合測定法及びＳＶ４０Ｔ抗原結合測定法を実施した。

【００７７】免疫親和カラムの溶離画分の全タンパク質の量をマイクロ－ＢＣＡ測定法（ＰＩＥＲＣＥ）により求めた。溶離タンパク質サンプルを次にＳＤＳ－ＰＡＧＥにより分析し、そして溶離物のＲＢタンパク質の量をクマシーブリリアントブルー染色法、次に濃度測定法により求めた。細胞抽出物の全タンパク質量はブラッドフォードの方法（Ｂｉｏ－Ｒａｄ）により測定した。［Ａｎａｌ．Ｂｉｏｃｈｅｍ．、７２：２４８－２５４（１９７６）］。細胞溶解産物のＲＢタンパク質を定量するために、標準として順に希釈した精製ＲＢタンパク質を使用して、ウエスタンブロッティングを実施してから、バンド強度について濃度測定により比較した。この点については、表１を参照すればよい。

【００７８】常法によりタンパク質ブロッティングを実施した。ボウエン（Ｂｏｗｅｎ）等の記載に従ってブロットを放射線標識化ＤＮＡによりインキュベーションした。［Ｎｕｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．８：１－２１（１９８０）］。これは室温で行った。ブロットを素早く水洗してから、６Ｍの尿素及び０．２％のＮＰ－４０（いずれも２０分）で３度洗浄し、引き続き、ＤＮＡ結合緩衝液（１０ｍＭのトリス－ＨＣｌ、ｐＨ７．０；１ｍＭのＥＤＴＡ；５０ｍＭのＮａＣｌ；０．２％のＢＳＡ；０．２％のＦｉｃｏｌｌ４００及び０．２％のポリビニルピロリン）で４回洗浄（いずれも３０分）した。次に、^{32}Ｐ－標識化ＤＮＡを含むＤＮＡ結合緩衝液中で３０分間ブロットをインキュベーションした。ＥｃｏＲ１で線状化したｐＧＥＭ１ＤＮＡをランダムプライミングによりα－^{32}Ｐデオキシヌクレオチド（Ａｍｅｒｓｈａｍ、＞３０００Ｃｉ／ｍｍｏｌ）で標識化し、プローブとして使用した。交雑後、ブロットをＤＮＡ結合緩衝液で３回（いずれも１０分）洗浄し、風乾し、ラジオオートグラフィーによって分析した。対照としてＴｒｐＥ－ＲＢ融合タンパク質を含めた。タンパク質が含むＲＢ遺伝子のエクソンに従い各ｔｒｐＥ－ＲＢ融合タンパク質を命名した。すなわち、ＲＢ１９－２２、ＲＢ２３－２７及びＲＢ１９－２７はそれぞれエクソン１９－２２（アミノ酸６１２－７７５）、エクソン２３－２７（アミノ酸７７６－９２８）及びエクソン１９－２７（アミノ酸６１２－９２８）のｐｐ１１０^{RB}領域を占める。

【００７９】Ａｄ－ＳＶＸ１－感染２９３細胞からＳＶ４０Ｔ抗原を免疫クロマトグラフィー精製した。［Ｊ．Ｖｉｒｏｌ．、５３：１００１－１００４（１９８５）；Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ　Ｐｒｅｓｓ．、Ｃｏｌｄ　ＳｐｒｉｎｇＨａｒｂｏｒ、ＮＹ、ｐｐ．１８７－１９２（１９８２）］。そして、抗－Ｔ単クローン性ＰＡＧ４１９抗原をＯｎｃｏｇｅｎｅ社から得た。公知複合体形成測定法を実施した。但し、若干の変更を加えた。即ち、８００ｎｇのバキュロウィルス発現ＲＢタンパク質を１ｍＭのＰＭＳＦ、２５μｇ／ｍｌのロイペプチン及び５０単位／ｍｌのアプロチニンを含む１ｍｌのＥＢＤ緩衝液（５０ｍＭのトリス－ＨＣｌ、ｐＨ８．０；１２０ｍＭのＮａＣｌ及び０．５％のノニデットＰ－４０）と混合した。混合物に８００ｎｇの精製Ｔを加え、９０分間氷上でインキュベーションした。抗－ＲＢ０．４７又はＰＡＢ４１９抗原のいずれかを用いて混合物のアリコットを免疫沈降し、ウエスタンブロッティング分析した。ブロットは順にｐＭＧ３－２４５、ＰＡＢ４１９と反応させた。アルカリ性ホスファターゼ接合ヤギ抗－マウスＩｇＧでインキュベーションした後、発色性基質を用いてブロットを発色処理した。

【００８０】精製ＲＢタンパク質がＳＶ４０Ｔ抗原と特異的複合体を形成できる能力をテストするために、等量のＲＢタンパク質とＴ抗原を混合し、抗－ＲＢ０．４７抗体か抗－Ｔ抗原ＰＡＢ４１９のいずれかを用いて、混合物のアリコットを免疫沈降した。

【００８１】この点に関連して、図１０にバキュロウィルス発現ＲＢタンパク質とＳＶ４０Ｔ抗原とが形成する複合体を示す。インビトロで精製バキュロウィルス発現ＲＢタンパク質を精製Ｔ抗原と混合した。次に、同アリコットの混合物をＰＡＢ４１９（経路２）か抗－ＲＢ０．４７（経路３）のいずれかで免疫沈降し、ウエスタンブロッティングにより分析した。経路１及び４はそれぞれＰＡＢ４１９で免疫沈降した精製ＳＶ４０Ｔ抗原、及び抗－ＲＢ０．４７抗体で免疫沈降した精製バキュロウィルス発現ＲＢタンパク質を示す。

【００８２】図１０に示すように、インビトロでＲＢタンパク質とＴ抗原を混合すると、ＲＢタンパク質とＰＡＢ４１９が共免疫沈降するだけでなく（経路２）、Ｔと抗－ＲＢ０．４７抗原が共免疫沈降した（経路３）。これらデータはバキュロウィルス感染昆虫細胞からのＲＢタンパク質がＳＶ４０Ｔ抗原と特異的複合体を形成できることを実証している。

【００８３】Ｂ６．精製ＲＢタンパク質の核転移

精製タンパク質がインビトロでＲＢの２つの公知生化学的特性をもつことが判明したので、次に精製タンパク質のインビトロ挙動について調べた。エクソン２１－２７のない、欠損ＲＢ遺伝子を含み、かつＣ末端切断ＲＢタンパク質（ｐ９５）をコード化する骨肉腫細胞系とのＳａｏｓ－２細胞の細胞形質に精製ＲＢタンパク質を注射した。［Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．Ｕ．Ｓ．Ａ．、８７：６－１０（１９９０）］。抗体はＲＢタンパク質のＣ末端に向かう事実を考慮して、使用する

抗－ＲＢ０．４７抗体によって認められない程度の少量で細胞形質にこのタンパク質を定位した。注射直後に、細胞を固定し、免疫染色法によって分析した。図１１にＳａｏｓ－２細胞の細胞形質へ顕微注射した後の生成ＲＢタンパク質の核定位を示す。細胞に生成ＲＢタンパク質を注射し、免疫染色法により分析した。図中、矢印は非注射細胞に比べて、顕微注射後の核の染色が強いことを示している。

【００８４】図１１に示すように、非注射細胞に比べて、顕微注射後の核の染色が強く（矢印）、注射タンパク質が核に直ちに輸送されたことを示す。ＲＢタンパク質は核タンパク質として知られているので、顕微注射後、精製タンパク質の核定位が直ちに、そして正確におきるが、これはこのタンパク質がインビボで活性なことを示唆している。

【００８５】顕微注射の場合、２０mMのトリスーＨＣｌ、ｐＨ７．４；１０mMのＫＣｌ；０．１mMのＥＤＴＡ；０．１mMのＤＴＴ及び２％のグリセロールを含む注射緩衝液に最終濃度が０．５ｍｇ／ｍｌになるように精製ＲＢタンパク質を透析した。ガラス毛管針（Ｅｐｐｅｎｄｏｒｆ社）を使用して、ガラスチャンンバースライド上に成長したＳａｏｓ－２細胞を通常の方法により顕微注射した。真空加圧装置を取り付けたＥｐｐｅｎｄｏｒｆ社のマイクロマニュピレーター及び逆相コントラスト顕微鏡（ニコン製）を使用して、それぞれ毛管を顕微操作し、そして顕微注射過程を視覚化した。顕微注射後、直ちに４％ホルムアルデヒドの０．０４Ｍリン酸緩衝液溶液（ｐＨ７．４）で細胞を固定し、免疫染色法により分析した。

【００８６】Ｂ６．要約

以上説明したように、バキュロウィルスポリヘドリンプロモーターの転写制御の下ヒト網膜芽腫遺伝子産生物が効率的に発現できることが証明できた。ＲＢタンパク質は細胞の成長を妨害するだけでなく、場合によってはそれに対して“毒性”を示す疑いがあったため、長い間、高いレベルでＲＢタンパク質を発現する試みは難しいと考えられていた。ポリヘドリンプロモーターからの外来遺伝子の転写は感染の後半で生じ、この後に細胞外ウィルス粒子の産生、そして細胞及び大半のウィルス遺伝子の遮断が続く。従って、過剰産生した場合細胞成長に有害なことがあるＲＢタンパク質等のタンパク質の合成にバキュロウィルス昆虫細胞系は有利である。この系の別な長所は昆虫及び哺乳動物細胞のタンパク質合成経路の類似性にある。

【００８７】また、ＲＢタンパク質は昆虫細胞の核に正確に到達することも判明し、これは哺乳動物の核定位信号が昆虫細胞により認識されることを含意している。バキュロウィルス発現系における組換えタンパク質のグリコシル化は高マンノース型のＯ－結合及びＮ－結合オリゴ糖に制限されているが、外来タンパク質の適当なホスホリル化はｃ－ｍｙｃ及びＨＴＬＶ－Ｉｐ40ˣの発現について報告されている（Ｊ．Ｖｉｒｏｌ．）。ＲＢタンパク質は以前からホスホリル化するが、グリコシル化はしないことが知られていた。これがバキュロウィルス発現系が機能性ＲＢタンパク質の産生に好適な理由である。

【００８８】本明細書に開示したように、感染昆虫細胞に産生したＲＢタンパク質は翻訳後にホスホリル化し、従って真正な哺乳動物のＲＢタンパク質の場合と全く同様に、ウエスタンブロッティング分析によって多重バンドを分化できる。ところが、バンド強度からみれば、ハイパーホスホリル化ＲＢタンパク質と比較した場合、未ホスホリル化及びホスホリル化形態が優勢である。現在までのところ、この現象がウィルス性溶解感染時の母集団の細胞周期状態の反映なのか、あるいは多量の外生ＲＢが細胞に存在するため、昆虫キナーゼによるタンパク質のホスホリル化が単に不十分なためなのかは知られていない。ホスホリル化パターンガ哺乳動物タンパク質の場合と正確に同じなのかをを決定するためには、ＲＢタンパク質のホスホリル化位置を精密にマッピングする必要がある。

【００８９】バキュロウィルス系に発現した組換えＲＢタンパク質の全レベルは感染昆虫細胞培養物（10^9細胞）１リットルにつき約１７－１８ｍｇである。この発現レベルはこの系が発生する他の哺乳動物タンパク質に相当する。例えば、インターロイキン２の場合は１０－２０ｍｇ／ｌである［Ｔｈｅ　Ｂａｎｂｕｒｙ　Ｒｅｐｏｒｔ．Ｆｉｅｌｄｓ，Ｂ．，Ｍａｒｔｉｎ，Ｍ．Ａ．＆　Ｋａｍｌｅｙ，Ｄ．（ｅｄ．）、２２：３１９－３２８（１９８５）、Ｃｏｌｄ　ＳｐｒｉｎｇＨａｒｂｏｒ　Ｌａｂｏｒａｔｏｒｙ　Ｐｒｅｓｓ、ＣｏｌｄＳｐｒｉｎｇＨａｒｂｏｒ］。また、Ｐ２１０ＢＣＲ－ＡＢＬの場合には、４－５ｍｇ／ｌである［Ｏｎｃｏｇｅｎｅ、４：７５９－７６６（１９８９）］．その５’非コード化領域の大部分を取り去ったＲＢｃＤＮＡに融合した、欠損のないポリヘドリン５’未翻訳領域を含む組換え転移ベクターを使用すれば、高ＲＢタンパク質発現レベルを更に高くすることができる。このＲＢｍＲＮＡ配列は高度にＧ＋Ｃリッチで、安定な２次構造を形成するのに有利なファクターである。これら構造体については、開始コドンの前にある時、対応ｍＲＮＡの翻訳効率を低くするものと考えられる。ＲＢｍＲＮＡのインビトロ翻訳については、ＲＢ５’未翻訳配列をアルファルファモザイクウィルス（ＡＭＶ）ＲＮＡ４又はβ－グロブリンｍＲＮＡのそれと置換することにより５倍から１０倍高くなることが実証され、これはＲＢ５’非コード化配列の翻訳に潜在的に悪影響を及ぼすことを示唆するものである［ＥＭＢＯ　Ｊ．（１９９０）］。また、外来遺伝子の長い５’未翻訳配列が存在すると、バキュロウィルス系における組換えポリヘドリン発現に影響するこ

とも判明している。ポリヘドリンプロモーターはＡ＋Ｔリッチ性が非常に強いため、結論としては、転移ベクターに挿入する前に、長く、かつＧ＋Ｃリッチな5’非コード化配列をＲＢｃＤＮＡからトリミングして、ｐｐ１１０^{RB}発現を最適化すべきである。

【００９０】精製中のタンパク質の変性を最小限に抑えるために、親和カラムからのＲＢタンパク質の溶離について何種類かの異なるプロトコルをテストした。ＲＢタンパク質の生物学的機能及び生化学的特性についてはそれ程多くは知られていないので、精製タンパク質の完全性の尺度として使用できるのは２つのパラメーターのみである。即ち、ＤＮＡ結合活性と、ＳＶ４０Ｔ抗原との複合体形成である。なお、タンパク質の生化学的特性を維持する条件は、本発明ではｐＨ１０．８で２０ｍＭのトリエチルアミンを使用する溶離条件であった。顕微注射後に細胞形質から精製タンパク質が直ちに核定位したが、これは該タンパク質がこの溶離条件下で活性を示すことを実証するものである。極端なｐＨ（２００ｍＭのグリシン、ｐＨ2，３、又は１００ｍＭのトリエチルアミン、ｐＨ１１．５）でタンパク質を溶離すると、タンパク質が変性する傾向が認められた。即ち、上記２つの活性が低下した。これは、長期間保存後に、不溶な凝集体が形成したことからも明らかである。

【００９１】以前の報告によれば、未ホスホルリル化ＲＢタンパク質のみがサルの腎細胞系統ＣＶ１－ＰのＳＶ４０Ｔ抗原による安定な形質転換株である、Ｄ２Ｃ２細胞のＳＶ４０Ｔ抗原を結合できるとされていたが［Ｃｅｌｌ、５６：５７－６５（１９８９）］、ＲＢタンパク質のある種のヒポホスホリル化形態もＳＶ４０Ｔ抗原と複合体を形成できることが見いだされた。これは、Ｔ抗原とＡｃＮＰＶ－Ｙ４ＲＢ感染昆虫細胞からの精製ＲＢタンパク質、あるいはモルトー４溶解産物とをインビトロ混合した場合に再現性よく確認できた。同じ現象は、Ｃｏｓ細胞を使用してインビボで複合体を形成した場合にも認められた（図１０）。ＲＢタンパク質のホスホリル化は相特異性の場合細胞周期中に変動し、かつＲＢとウィルス性腫瘍タンパク質の間の複合体形成はこれらＤＮＡ腫瘍ウィルスの形質転換活性に関与しているので、ヒポホスホリル化ＲＢタンパク質とＳＶ４０Ｔ抗原との関連性はいずれ解明すべきものである。

【００９２】以上説明したように、バキュロウィルス昆虫細胞系を使用すると、可溶な、欠損のない、活性をもつと予測できるＲＢタンパク質を有意味な量で使用できることは、ＲＢ遺伝子産生物の生化学的及び生物物理学的特性の将来の研究にとって大きな前進である。考えられるケースには、関連する細胞タンパク質の分析、これらが相互作用する特異的ＤＮＡ配列の分離やＸ線結晶学

を利用するＲＢタンパク質の３次元的構造研究等である。また、癌抑制における網膜芽腫遺伝子の生物学的機能の解明も進展するはずである。顕微注射により直接テストした、ＲＢの細胞成長及び分化への考えられる関与については、現在精力的に研究している。

【００９３】本明細書で使用した記号について以下説明しておく。ｃＤＮＡは相補ＤＮＡ、ｋｄはキロダルトン、ｋｂはキロ塩基、ＳＤＳは硫酸ドデシルナトリウム、ＰＡＧＥはポリアクリルアミドゲル電気泳動、ＮＰ－４０はノニデットＰ－４０、ＭＥＳは（２－［Ｎ－モルホリノ］エタンスルホン酸）ナトリウム塩、ＭＯＩは感染の多重度、Ｍｒは相対分子量、そしてＰＡＰはポテト酸ホスファターゼである。“ｐｐ１１０^{RB}”で示したタンパク質産生物は“ｐｐＲＢ^{110}”と同じタンパク質産生物である。

【００９４】本発明を特定実施例について説明してきたが、各種の変更が可能であり、いずれも請求項に記載した精神及び範囲に包含されるものである。即ち、本開示は制限を意図するものではない。

【図面の簡単な説明】

【図１】ｐｐ１１０RB合成するバキュロウィルス発現ベクターの構成を示す概略図である。

【図２】ｐｐＲＢ感染昆虫細胞のウエスタンブロットである。

【図３】感染から７２時間後までに感染細胞から抽出した細胞抽出物を同定するウエスタンブロットである。

【図４】ＲＢタンパク質の細胞間定位を示す顕微鏡写真である。

【図５】感染Ｓｆ９細胞を示す顕微鏡写真である。

【図６】昆虫細胞におけるＲＢタンパク質のホスホリル化及び脱ホスホリル化分析結果を示すラジオオートグラフである。

【図７】ｐＭＧ３－２４５抗－ＲＢからの粗溶解産物、感染Ｓｆ９細胞及び溶離物の電気泳動分析を示す。

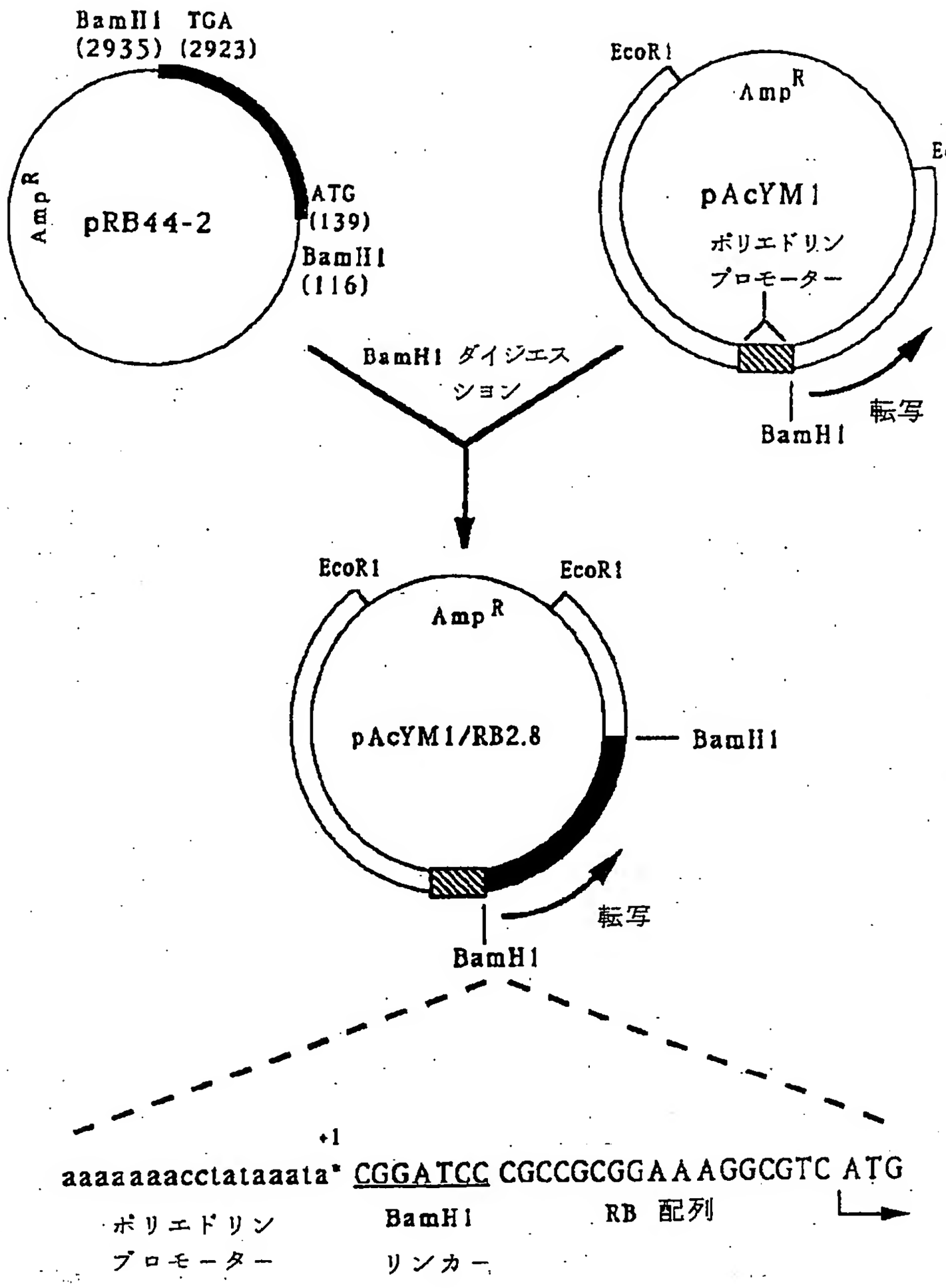
【図８】１０％ＳＤＳ－ＰＡＧＥ、クマシーブリリアントブルー染色法に適用した融合タンパク質及びバキュロウィルス発現ｐｐ１１０RBのサウスウエスタンＤＮＡ結合測定法を示す。

【図９】図８を作成するために使用したゲルに対する平行ゲルからのブロットのラジオオートグラフであり、該ブロットを32Ｐ－標識化ＤＮＡフラグメントでインキュベーションしたものである。

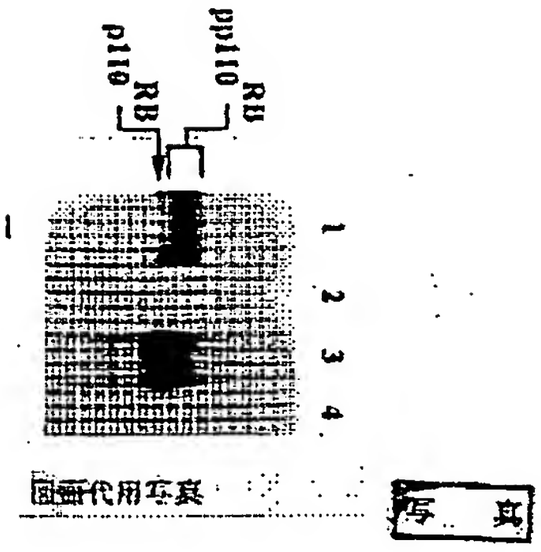
【図１０】バキュロウィルス発現ＲＢタンパク質とＳＶ４０Ｔ抗原との複合体形成を示すクロマトグラムである。

【図１１】Ｓａｏｓ－２細胞の細胞軽質への顕微注射後の精製ＲＢタンパク質核定位を示す写真である。

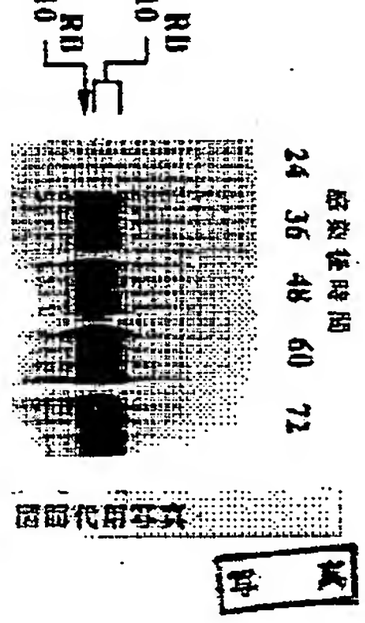
【図１】



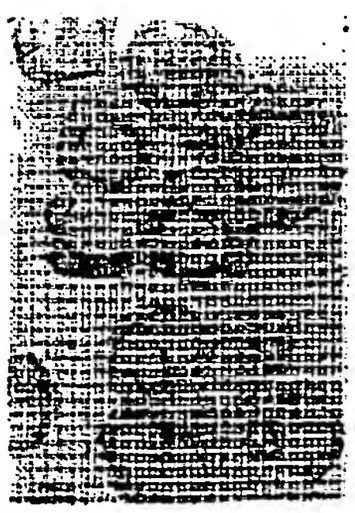
【図２】



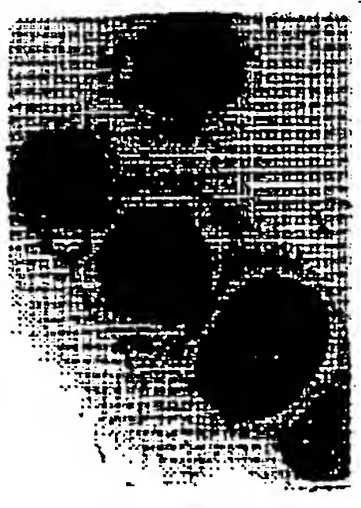
【図３】



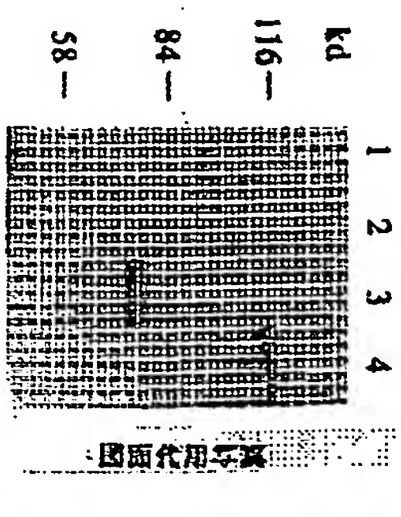
【図４】



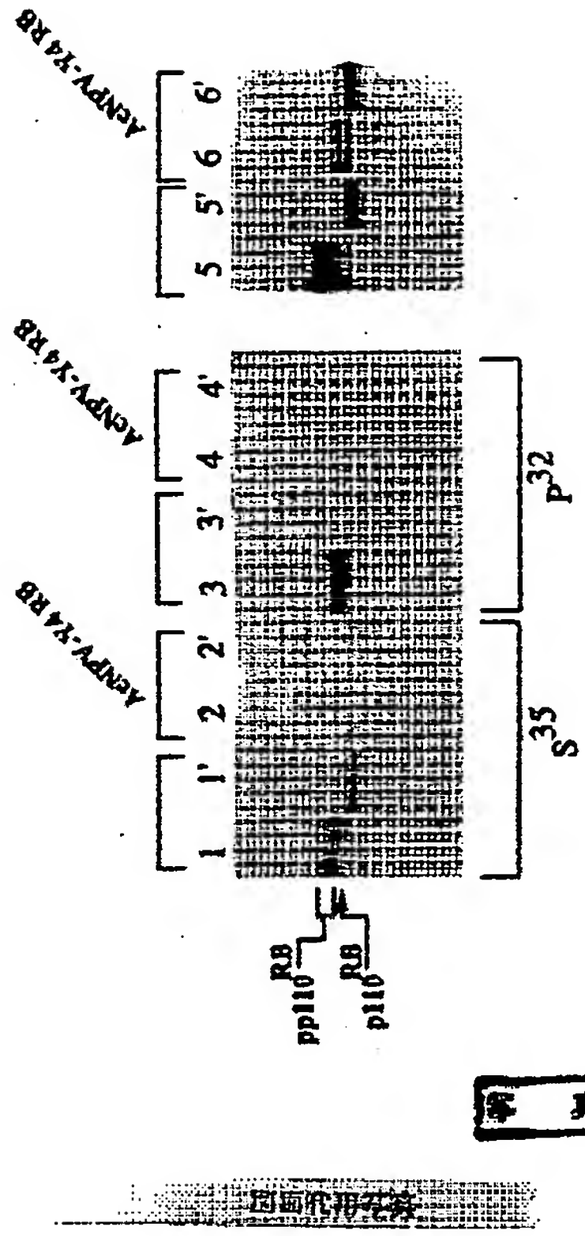
【図５】



【図８】

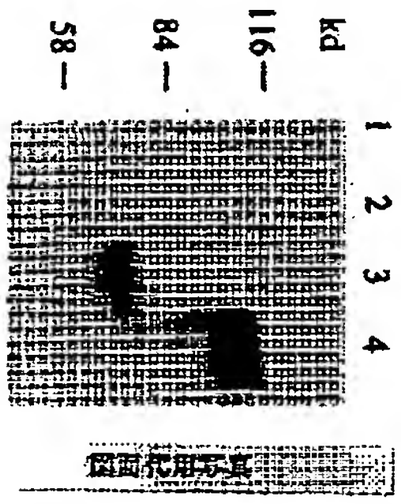


【図６】

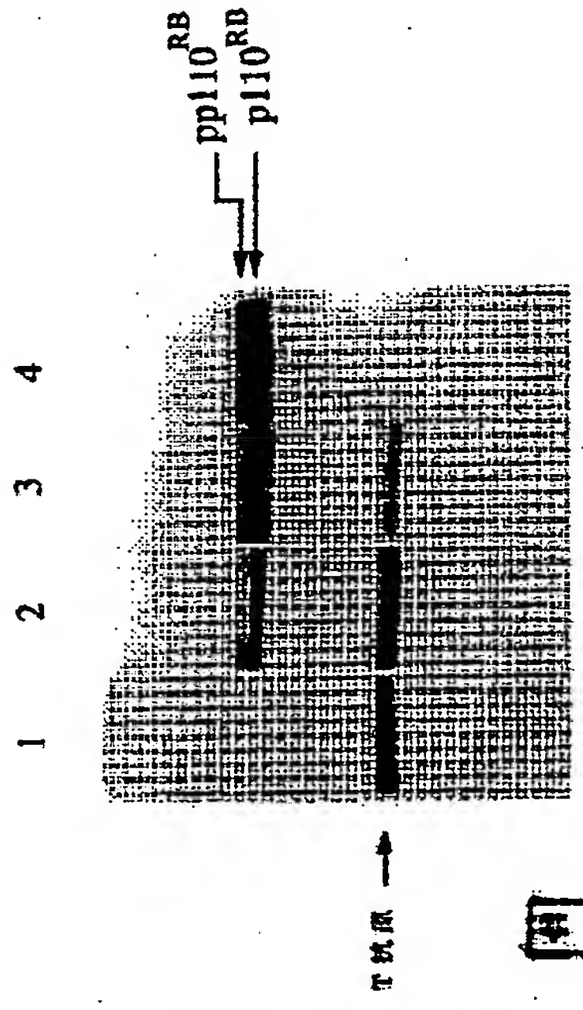


【図７】

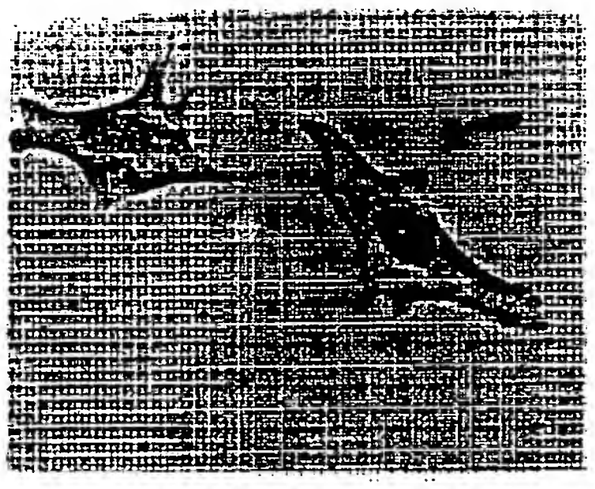
【図９】



【図１０】



【図１１】



【手続補正書】
【提出日】平成５年８月１８日
【手続補正１】
【補正対象書類名】明細書
【補正対象項目名】図面の簡単な説明
【補正方法】変更
【補正内容】
【図面の簡単な説明】
【図１】ｐｐ１１０ＲＢ合成するバキュロウィルス発現ベクターの構成を示す概略図である。
【図２】ｐｐＲＢ感染昆虫細胞のウエスタンブロット写真である。
【図３】感染から７２時間後までに感染細胞から抽出した細胞抽出物を同定するウエスタンブロット写真である。
【図４】ＲＢタンパク質の細胞間定位を示す顕微鏡写真である。
【図５】感染Ｓｆ９細胞を示す顕微鏡写真である。
【図６】昆虫細胞におけるＲＢタンパク質のホスホリル化及び脱ホスホリル化分析結果を示すラジオオートグラフを示す写真である。
【図７】ｐＭＧ３－２４５抗－ＲＢからの粗溶解産物、感染Ｓｆ９細胞及び溶離物の電気泳動分析を示す写真である。
【図８】１０％ＳＤＳ－ＰＡＧＥ、クマシーブリリアントブルー感染法に適用した融合タンパク質及びバキュロウイルス発現ｐｐ１１０ＲＢのサウスウエスタンＤＮＡ結合測定法を示す写真である。
【図９】図８を作成するために使用したゲルに対する平行ゲルからのブロットのラジオオートグラフであり、該ブロットを３２Ｐ－標識化ＤＮＡフラグメントでインキュベーションした写真である。
【図１０】バキュロウィルス発現ＲＢタンパク質とＳＶ４０Ｔ抗原との複合体形成を示すクロマトグラフを示す写真である。
【図１１】Ｓａｏｓ－２細胞の細胞軽質への顕微注射後の精製ＲＢタンパク質核定位を示す写真である。

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